Order Number 9521272

Molecular genetic analysis of the functional domains of the MAL-activator of *Saccharomyces cerevisiae*

Gibson, Andrew Winston, Ph.D.

City University of New York, 1995

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

MOLECULAR GENETIC ANALYSIS OF THE FUNCTIONAL DOMAINS OF THE MAL-ACTIVATOR OF *SACCHAROMYCES CEREVISIAE*.

by

Andrew Winston Gibson

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1995

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

December 21, 1994
Date

Corinne A. Michels
Chairman of Examining Committee
Prof. Corinne Michels, Queens College

January 6, 1995
Date

Richard L. Chappell
Executive Officer
Dr. Richard L. Chappell

S. A. R. T.
Prof. Susan Rotenberg, Queens College

Wilma Saffran
Prof. Wilma Saffran, Queens College

Peter Lipke
Prof. Peter Lipke, Hunter College

Anne Draginis
Prof. Anne Draginis, St. Johns University

Supervising Committee

The City University of New York

ABSTRACT

A MOLECULAR GENETIC ANALYSIS OF THE FUNCTIONAL DOMAINS OF THE MAL63 ACTIVATOR OF *SACCHAROMYCES CEREVISIAE*

by

Andrew W. Gibson

Advisor: Dr. Corinne A. Michels

Maltose fermentation in *Saccharomyces cerevisiae* requires any one of five dominant, unlinked *MAL* loci (*MAL1, MAL2, MAL3, MAL4, MAL6*). Three genes are encoded at each locus: Gene 1 encodes maltose permease, a membrane protein which transports maltose across the plasma membrane; Gene 2 encodes maltase, an α-glucosidase which hydrolyzes maltose to give two glucose monomers; and Gene 3 which encodes the MAL-activator, a positive regulator of the expression of the structural genes.

MAL63, which encodes the activator at the *MAL6* locus, encodes a predicted 470 amino acid residue DNA-binding protein with a cysteine-rich zinc cluster (residues 8-38). A peptide containing this zinc cluster was shown to bind to the UAS_{MAL} in

the promoter region of the structural genes to activate their transcription.

To determine the other functional domains of the Mal63p activator, we carried out a deletion analysis using *LexA-MAL63* gene fusions. The analysis indicates that the DNA-binding domain of Mal63p extends to residue 100, that a transcription activation domain lies within residues 100-250, and that a maltose-responsive regulatory domain exists in residues 283-470. Our analysis also indicates that the fourteen C-terminal residues are required for function.

A molecular genetic analysis of the Mal43-Cp constitutive activator, encoded at the *MAL4* locus, showed that C-terminal alterations between residues 215-470 were responsible for its unregulated mutant phenotype. However, the phenotype of Mal43-Cp could not be limited to one or even a few of the 27 amino acid residue alterations in this region.

Overexpression of *MAL63* on a multi-copy plasmid failed to uncover the existence of any titratable Gal80p-like inhibitor of Mal63p activity.

A Two-Hybrid analysis, performed in order to test the hypothesis that different regions of Mal63p interacted revealed no such interaction.

A model of MAL-activator induction by maltose is discussed.

ACKNOWLEDGMENTS

I wish to thank my thesis advisor Dr. Corinne Michels for her expert guidance throughout this work, for recognizing a talent whose existence I wasn't sure of, and for initially planting the idea of doctoral study in my mind.

I wish to thank my committee members for their suggestions in this work and for volunteering their time to evaluate this work.

I wish to thank all of my colleagues and friends in our lab (Kim, Qi, Lori, Frank, Chantal, Han, Sara, Igor, Zhen, Li, Hua, Bin, Galo) and in the adjacent labs (Reggie, Terri, Nicos, Harleen, John) for all of our lunch-time discussions on every subject from Biology to Politics. Your friendship made the goodtimes better and the downtimes bearable.

I wish to thank the Faculty and Staff of the Biology Department for all their help and for their kind words of encouragement during my stay at Queens College.

Finally, I wish to thank my parents, Hazel and Creighton Gibson; my Grandparents, Vivienne and the late Winston DaCosta

Sealy with whom I spent most of my formative years; my siblings (and best friends), Marston (the Judge), Ian, Don, Patrick, Ann and Donna; my uncle (and surrogate father), Wendle Sealy; my aunt (and surrogate mother) Monica Sealy-Puckerin and the rest of my family whose love and support made all this happen.

TABLE OF CONTENTS PAGE

LIST OF TABLES PAGE

LIST OF FIGURES PAGE

INTRODUCTION

Eukaryotic cells respond to signals in their immediate environment (nutrients, hormones, growth factors, etc.), which result in physiological changes that modulate the expression of genes required for subsequent metabolic reactions. Increasing the expression of target genes above basal levels often requires the presence of regulatable, sequence-specific transcription activators which must enter to the nucleus, bind to upstream activating DNA sequences (UAS) linked to the target gene(s), and interact directly or indirectly with the pre-assembled RNA polymerase II-dependent transcriptional complex at the promoter (Stanway 1993; Tjian and Maniatis, 1994). In most cases regulation of the expression of the target genes appears to involve modulating the activity of the transcription activator by preventing or promoting its ability to localize to the nucleus, bind to promoter sequences, or activate transcription. Therefore, the transcriptional activator must have within its structure a region capable of responding to the presence of the regulatory signal, so that target gene expression can be rapidly achieved. Although there are examples of mechanisms which regulate

transcriptional activator activity by promoting or preventing nuclear localization or DNA-binding (Karin 1990), the mechanisms underlying regulation of the activation function of transcriptional activators remain unclear. This lack of information results, in part, from the complexity of the transcription activation process, and the limitations and difficulties encountered in working in mammalian or *in vitro* reconstitution systems (Jayaraman 1994).

In contrast to more complex systems, *Saccharomyces* yeasts have provided a more easily accessible system for identifying and cloning regulatory genes, and for functionally characterizing the gene products and mechanisms regulating their activity. The ease with which yeast cells and their genomes can be manipulated, the well established genetic system, and the fact that many of the factors and mechanisms uncovered find parallels in higher organisms make yeast an ideal system for understanding regulated gene expression.

Several yeast and mammalian transcriptional activators have been studied extensively (Guarente, 1984). Studies reveal that, to a first approximation, transcription activators are composed of domains representing separable functional units which together form a regulatable, fully functional activator. In the long run, though, this view may turn out to be too simplistic since recent evidence seems to indicate that transcription activators are more complex structures containing overlapping functional domains, and

sometimes require the integrity of larger physically distinct regions in order to function. In the following section I briefly review the regulation of some well studied eukaryotic transcription activators.

The Gal4-activator. *GAL4* encodes an 881 amino acid residue zinc- dependent DNA-binding protein, Gal4p, which regulates the expression of the genes required for galactose fermentation (Hopper *et al.*, 1978; Johnston and Hopper, 1982; Johnston 1987) by binding to distinct DNA sequences upstream of these target genes (Guarente *et al.*, 1982; Giniger *et al.*, 1985) and making direct or indirect contact with the transcription machinery at the promoter (Johnston 1987; Lewin 1990). Expression of the *GAL* structural genes is induced 1000 fold by galactose or a galactose metabolite, and is repressed in the presence of glucose (Oshima, 1982; Adams, 1973). The ability of Gal4p to activate transcription of the target genes is regulated by the inhibitory protein, Gal80p (Johnston 1987). In the absence of the inducer, galactose, Gal4p activity is inhibited by physical interaction with Gal80p (Johnston *et al.*, 1987). In the presence of galactose the inhibition is relieved. The mechanism of this inhibitory interaction is believed to involve allosteric changes in the Gal4p/Gal80p complex. Overexpression of *GAL4* on a high copy number plasmid (Johnston and Hopper, 1982), or overexpression of the C-terminal 30 residues (Ma and Ptashne, 1987b) both

relieve Gal4p of inhibition by Gal80p and result in constitutive, glucose-repression-insensitive expression of the target genes. This suggests that the Gal80p inhibitor is present in limiting quantities.

The Gal4p activator protein contains a DNA-binding domain and nuclear targeting signal between residues 1-147, and two acidic transcription activation domains; a cryptic activation domain between residues 148-196 and the major activation domain between residues 768-881 (Johnston 1987). Recent experiments suggest that the acidic residues of the activation domain of Gal4p, which were thought to form an α-helical structure crucial to its function, do not require the acidic character of the residues for transcription activation, and do not form an α-helical structure in solution (Leuther *et al.*, 1994). Using site directed mutagenesis the acidic residues, 870-873(DDED), were deleted or replaced with basic residues (Leuther *et al.*, 1994). In both cases, Gal4p was still transcriptionally active. Using circular dichroism spectroscopy it was shown that, at pH6, synthetic peptides containing the Gal4p activation domain form a β-sheet instead of an α-helix (Van Hoy *et al.*, 1994). In addition, alterations in residues D862 and D863 resulted in a transcriptionally active Gal4p which no longer interacted with Gal80p. Therefore, these acidic residues are critical for Gal4p/Gal80p interaction only.

The Gal4p domain required for interaction with Gal80p has

been localized to the C-terminal 30 amino acid residues, which are also required for transcription activation (Ma *et al.*, 1987b). Constitutive mutations map to this 30 amino acid residue C-terminal region in Gal4p, and to Gal80p (Salmeron *et al.*, 1990). The co-localization of the transcription activation domain and the Gal80p binding domain to the same C-terminal residues of Gal4p suggest that inhibition of Gal4p activity may involve steric occlusion of its activation domain by Gal80p in the absence of inducer. In the presence of the inducer, a conformational change is believed to occur in the Gal4/Gal80 complex, rather than a separation of the two proteins. This stems from studies which indicate that the Gal4p/Gal80p complex forms under all conditions (Johnston *et al.*, 1987), and is always bound to the UAS_{GAL} (except in the presence of glucose). More recent experiments have confirmed the stability of the Gal4p/Gal80p complex, which can be precipitated from cells even after induction by galactose (Leuther and Johnston, 1992). In addition, a phosphorylated form of Gal4p exists in the transcriptionally active Gal4/Gal80p complex (Parthum and Jaehning, 1992). Previously, three different phosphorylated forms of Gal4p, two of which are more heavily phosphorylated, had been isolated (Mylin *et al.* 1989). The two more heavily phosphorylated forms appeared to correlate with higher rates of transcription activation by Gal4p, and were seen in the presence of inducer, but not in the presence of glucose (Mylin *et al.*, 1990). The current

model suggests that galactose, or the inducer, signals a conformational change in the Gal4p/Gal80p complex, which now reveals the previously hidden Gal4p activation domain to the transcriptional machinery at the promoter.

The Hap1p activator. *HAP1* encodes a 1483 amino acid residue zinc dependent, heme regulated DNA-binding protein which regulates the expression of many mitochondrial enzymes involved in the oxidative-phosphorylation/electron transport pathways, including the two isozymes of cytochrome-c, iso-1-cytochrome-c (*CYC1*), and iso-2-cyctochrome-c (*CYC7*). In the presence of heme, Hap1p recognizes and binds, with equal avidity, to two distinctly different upstream activating sequences in the promoter regions of these genes (Pfeifer *et al.*, 1987). The DNA-binding and dimerization domains of Hap1 lie within residues 1-148 (Zhang *et al.*, 1993). An acidic activation domain lies between amino acid residues 1309 and 1483, and a heme binding domain lies between residues 144 and 444. An internal 800 residue region is of unknown function. C-terminally deleted mutant Hap1p proteins containing the first 444 amino acid residues bind to the UAS sequences of both *CYC1* and *CYC7* in a heme dependent manner. However, mutant proteins containing residues 1 to 144 bind independently of heme, and result in constitutive expression of *CYC1* and *CYC7* (Pfeiffer *et al.*, 1987). Deletion of the heme regulatory region alone from the wild type Hap1p protein

results in constitutive, heme independent expression of *CYC1* and *CYC7* (Pfeiffer *et al.*, 1989). Recent data suggests that Hap1p is constitutively nuclear, but not bound to DNA and exists in an inactive form in a high molecular weight complex with a cytoplasmic factor in the absence of heme (Fytlovich *et al.*, 1993; Zhang and Guarente, 1994). Release from this factor requires the presence of heme, and deletion of the Hap1p heme-binding site prevents its formation. Thus, heme is required to disperse the high molecular weight complex, releasing an active Hap1 protein which can now dimerize, bind to the UAS, and activate transcription (Zhang and Guarente, 1994). Hap1p activity is also positively regulated by *TUP1/SSN6*, which encodes a general repressor of transcription (Keleher *et al.*, 1992). Tup1p/Ssn6p was shown to repress transcription of many different sets of genes, including a-specific, haploid-specific and glucose-repressible genes, when recruited by promoter-specific DNA-binding proteins. Since Tup1p/Ssn6p protein is not found in a complex with Hap1p, and since deletion of the heme-binding domain inhibits the formation of the Hap1p high molecular weight complex, it is suggested that the Tup1p/Ssn6p repressor does not interact with Hap1p directly, and that in the presence of heme it might repress the expression of a cellular factor which interacts with Hap1p. By lowering the free concentration of this cellular factor, formation of heme-bound active Hap1p is favored.

HAP1-18, which encodes a constitutive allele containing a Ser to Arg mutation in residue 63 adjacent to the zinc finger DNA-binding domain, is unable to bind to the UAS of *CYC1*, but increases the expression of *CYC7* (Kim and Guarente, 1989). Since the Hap1-18p mutant activator binds to the UAS of *CYC7* with the same affinity as the wild type protein, this suggests that the DNA-binding domain of Hap1p also affects its ability to activate transcription (Kim and Guarente, 1989). More recently, Hap1p has been shown to have novel elaborate functions in the expression of two other mitochondrial genes, *HEM13* and *ERG11* (Defranoux, *et al.*, 1994). While *HEM13*, encoding the major species of cytochrome P450, is expressed in the presence of high partial pressures of oxygen (when heme concentrations are high), *ERG11*, which encodes coproporphyrinogen oxidase, is preferentially expressed in low concentrations of oxygen. Using site-directed mutagenesis to alter Ser63 to Gly, Ile or Arg, it was shown that the expression of these two genes was altered differentially, both in the presence and absence of heme. In the absence of heme (low oxygen) the Gly63 and Ile63 mutations activate *HEM13* expression, but fail to activate expression of *ERG11*. The Arg63 mutation was a poor activator under these conditions. In the presence of heme (high oxygen), Gly63 and Ile63 activate *CYC3*, but not *CYC1* expression, while Arg63 activates *CYC3*, but not *CYC1* expression (Defranoux, *et al.*, 1994). This suggests that the DNA-binding, zinc cluster is also involved

in regulation of all target genes both in the presence and absence of heme, and that the regulatory function can be positive or negative for a particular gene. Since the same zinc cluster is involved in differential regulation of transcription, these data also suggest that another domain within Hap1p, or Hap1p interacting with another factor, regulates this difference in activation at the promoters of these genes. The result is a modification of the recognition specificity of Hap1p, or a modification of its efficiency in activating expression of the different target genes, and Ser63 may be part of a sub-region, within the zinc cluster, that is involved in the differential regulation of Hap1p transcriptional activity.

The Adr1p activator. Studies of regulatory mutations show that expression of the *ADH2* gene, which encodes glucose repressible alcohol dehydrogenase, is regulated by positive and negative regulators (Denis *et al.*, 1981; Denis 1984). Alcohol dehydrogenase converts ethanol to acetaldehyde and its expression is increased 200 fold during growth on ethanol, or when glucose in the media is exhausted. The best studied regulator of *ADH2* expression is Adr1p, encoded by *ADR1*. Adr1p is a 1323 amino acid residue, zinc-dependent positive activator which binds to two UAS regions, UAS1 and UAS2, upstream of *ADH2* (Ciriacy, 1979; Beier and Young, 1982). Overexpression of *ADR1* on a high copy number plasmid results

in constitutive expression of *ADH2* in a glucose insensitive manner (Denis, 1987; Blumberg *et al.*, 1988), indicating the presence, in limiting quantities, of a negative regulator. *ADR1* is constitutively expressed (Bemis *et al.*, 1988) and localized in the nucleus (Blumberg *et al.*, 1987). Two Adr1p monomers have been shown to bind, *in vitro*, to the UAS1 in the presence and absence of glucose (Bemis *et al.*, 1988; Thukral *et al.*, 1991). However, dimerization of Adr1p has not been demonstrated. A nuclear targetting signal exists in the first 16 residues (Blumberg *et al.*, 1987), and a zinc-finger DNA-binding domain lies within residues 99-155 of Adr1p.

Regulation of Adr1p activity is thought to occur by phosphorylation of the UAS-bound protein. Unregulated cyclic-AMP dependent protein kinase (cAPK) activity inhibited the transactivating ability of Adr1p *in vivo* (Taylor *et al.*, 1990). Site-directed mutagenesis and phosphopeptide mapping had shown that phosphorylation occurred on serine residue 230 and in another region N-terminal to Ser230 (Cherry *et al.*, 1989). In addition, mutation of residues 228 to 231, which lie within a cAPK recognition site, RRASFSG, resulted in constitutive, partially glucose-insensitive expression of *ADH2* (Taylor *et al.*, 1990; Cherry *et al.*, 1989). However, other sites independent of cAPK, are thought to be involved in the regulation of Adr1p activity since deletion of the cAPK recognition site (including serine 230) did not result in full insensitivity to glucose repression. In addition, two other

protein kinases, encoded by *SNF1* and *SCH9*, affect *ADH2* expression independently of both Adr1p and cAPK (Denis and Audino, 1991) and all cAPK effects were Adr1p dependent (Denis *et al.*, 1994). 21 *ADR1*^{c} mutations have been isolated and characterized. All mutations mapped to the cAPK region between residues 222-234. Studies of the cAPK-dependent phosphorylation pattern of synthetic peptides carrying alterations corresponding to each of the constitutive mutations revealed that only a subset of the changes interfered with the ability of cAPK to phosphorylate Ser230 (Denis *et al.*, 1994). A mutation converting Ser230 to a non-phosphorylatable residue was constitutive, and gave even higher levels of *ADH2* expression in the absence of cAMP. In addition, strains carrying deletions in two of the cAPK catalytic genes, *tpk1* and *tpk2*, and a *tpk3*^{ts} allele, and which expressed low levels of cAMP, did not have an *ADR1*^{c} phenotype. Taken together, these data suggest that cAPK acts through more than one mechanism to regulate *ADH2* expression, and that one of these mechanisms is independent of the effects of Ser230 phosphorylation (Denis *et al.*, 1992).

Using LexA/Adr1p hybrid derivatives, deletion analysis of Adr1p has shown that the activator is a complex protein containing functionally redundant domains (Cook *et al.*, 1994). Assays of the ability of LexA/Adr1p deletion hybrids to activate expression of a *LacZ* reporter gene under the control of a LexA-operator site revealed that Adr1p contains three

transcription activation domains (TAD); TADI (76-172), TADII (262-288) and TADIII (330-507). Two inhibitory regions, 148-263 and 220-263, when deleted individually from Adr1p, resulted in enhanced activity. The latter region, 220-263, contains the cAPK site, or *ADR1*^{c} region (227-239). Deletion of this region enhanced the activation of *LacZ* expression by all three TADs independently (Denis *et al.*, 1992). In addition, 220-260 inhibited the transactivation function of a synthetic heterologous activation domain fused to LexA (Denis *et al.*, 1992). Since no homology exists between Adr1p and the heterologous activation domain it is unlikely that similar inhibitory interactions occur between 220-260 and another region of Adr1p, and between 220-260 and the heterologous activation domain. A more likely explanation is that the 220-260 inhibitory region functions by binding to a cytoplasmic inhibitor. Therefore, the mechanism underlying regulation of Adr1p activity appears to involve a cytoplasmic inhibitory factor which has so far evaded detection by several mutagenic screens (Ciriacy, 1979; Denis, 1987). The data above also argue against a previous model which suggested that Adr1p activity is regulated by steric interaction between the inhibitory region (227-239) and a second region within Adr1p, and that the steric interaction prevented the Adr1p transcription activation domain from making contact with the general transcription machinery.

Recent data have added another level of complexity to the

regulation of Adr1p transcriptional activity. Seven intragenic suppressor mutations, which suppressed the constitutive, glucose-repression insensitive phenotype of *ADR1-5*^{c} were isolated (Cook *et al.*, 1994). All of these mutations occurred within the zinc-finger, DNA-binding region of the protein (97-155). When tested for their ability to bind to UAS1 in gel mobility shift assays, all mutations appeared to be deficient in their ability to bind DNA, and western blot analysis confirmed that all mutant alleles were expressed to levels similar to wild type *ADR1*. However, while a wildtype LexA/Adr1p hybrid could activate transcription of a *LacZ* reporter gene under the control of a LexA operator, similar LexA hybrid derivatives of the mutant alleles were severely deficient in their ability to transactivate. This indicates that mutations in the zinc-finger, DNA-binding domain of Adr1p also affect transcription activation, and is another example of DNA-binding domains that are also involved in transcription activation (see section on Hap1p).

The Leu3p activator. In the leucine biosynthetic pathway transcription of the *LEU1*, *LEU2* and *LEU4* structural genes is regulated by the Leu3p transcriptional activator, an 886 amino acid residue protein encoded by the *LEU3* gene. In the absence of the inducer, α-isopropylmalate, Leu3p binds to a sequence, UAS_L, upstream of the biosynthetic genes (Zhou and Kohlhaw, 1990; Sze *et al.*, 1992) and other yeast genes, but represses

transcription (Sze *et al.*, 1992). However, Leu3p activates expression of these genes only in the presence of α-isopropylmalate (Sze *et al.*, 1992). Sequence analysis shows that Leu3p has an amino-terminal, cysteine-rich DNA-binding domain (residues 1-173), which requires other internal regions of the protein for optimal binding, and a carboxy-terminal acidic transcription activation region (residues 855-886) (Zhou *et al.*, 1990). A Gal4p hybrid fusion to the C-terminal 30 residues of Leu3p activates expression of a reporter gene downstream of 5 Gal4p binding sites, and mutant Leu3p proteins, which lacked these C-terminal activation residues, were effective DNA-bound transcription repressors (Sze *et al.*, 1993). Cells overexpressing *LEU3* on a high copy number plasmid grew slowly, but did not show constitutive expression of *LEU1* and *LEU2* (Brisco *et al.*, 1987; Zhou *et al.*, 1990), suggesting that no titratable negative regulator, comparable to Gal80p, exists in this system. Internal deletions of an approximate 600 residue region between the two functional terminal regions of Leu3p resulted in unregulated Leu3p activity and constitutive expression of *LEU1* and *LEU2*. This indicates that the central region mediates a regulatory function in response to the inducer (Zhou *et al.*, 1990). Deletion of 79 residues within the inducer-responsive region, but immediately N-terminal to the activation domain, resulted in a loss of activation and inability to respond to inducer. Since the activation domain in this mutant allele is intact,

this suggests that the protein is in a conformation unfavorable for activation (Zhou and Kohlhaw, 1990). Taken together with the fact that Leu3p binds to UAS_L under all conditions, and that *LEU3* overexpression studies failed to uncover an inhibitor protein, these data suggest that the α-isopropylmalate inducer signals an activating allosteric change in the UAS-bound, inactive protein. In addition, three independent point mutations, which convert Trp861, Trp864 and Trp870 to alanine residues in the activation domain, resulted in activators with varying sensitivity to the inducer (Zhou *et al.*, 1990). The W864A allele resulted in a constitutive, super-inducible activator, while the W861A and W870A alleles were less affected. This suggests that these Trp residues in the Leu3p activation domain may make contact with the internal inducer-responsive domain. The current model for regulation of Leu3p activity suggests that in the absence of the inducer, the activation domain of the DNA-bound protein is hidden by hydrophobic interaction with the internal regulatory region, and that interaction of this region with the inducer causes an allosteric conformation change which results in the release of the activation domain from inhibition (Zhou and Kohlhaw, 1990).

The PHO4 activator. When the level of inorganic phosphate (Pi) in the medium is low cells derepress the synthesis of repressible acid phosphatase (rAPase) encoded by *PHO5, PHO10*

and *PHO11*(Thill *et al.*, 1983). Expression of these genes is repressed when Pi concentration is high (Lemire *et al.*, 1985). Genetic studies have identified 5 genes that regulate expression of the rAPase genes: *PHO2, PHO4* and *PHO81* are positive regulators, and *PHO80* and *PHO85* negatively regulate the rAPase genes. Of these regulators, the Pho4p activator has been well characterized (Lemire *et al.*, 1985). Overexpression of *PHO4*, which is constitutively expressed in the cell, results in constitutive expression of the rAPase genes under repressing conditions (high Pi), and higher than normal levels of expression under inducing conditions (low Pi)(Yoshida *et al.*, 1989). Pho4p protein contains an amino-terminal acidic transcriptional activation domain (residues 75 to 99), and a carboxy-terminal helix-loop-helix DNA-binding domain in the C-terminal 84 residues (Ogawa, 1990; Jayaraman, *et al.*, 1994). The 312 amino acid residue protein is believed to dimerize to form a functional activator, and the dimerization domain has been localized to residues 203 to 307 (Ogawa, 1990).

The activity of Pho4p is regulated by interaction with the inhibitor protein, Pho80p. Four dominant constitutive mutations, which alter proline residue 174 in *PHO4* have been isolated (Ogawa, 1990). In addition, a constitutive phenotype results when residues 163 to 171 are deleted or when four residues are inserted between residues 171 and 172. Mutations in this region are believed to alter an alpha-helical

structure, centered around Pro174, which is critical for interaction with negative regulators. Recently, suppressors of a dominant *PHO4*^{c} mutation have been isolated in *PHO80* (Okada and Toh-e, 1992). One dominant suppressor, *PHO80-2*, showed some allele specificity, suppressing the *PHO4*^{c}*-6* and *PHO4*^{c}*-d*(163-171) alleles, but not the *PHO4*^{c}*-d*(4-40) allele. These results are consistent with a model suggesting an inhibitory interaction between Pho4p and Pho80p. Using the Two-Hybrid System, it was shown that Pho4p interacts with Pho80p *in vivo*, and that the interaction does not involve the DNA-binding domain which is still functional (Jayaraman *et al.*, 1994; Fields and Song, 1987). Instead, interaction with Pho80p involved two domains of Pho4p, the N-terminal 31 residues, and residues 156-200. Interestingly, the transcription activation domain (75-99) lies between these two regions, and it is suggested that interaction with Pho80p inhibits Pho4p activity by physically masking the activation domain (Jayaraman, 1994). This type of masking effect has also been seen in the mammalian transcriptional activator, c-jun, in which the activation domain, a1, is masked by the interation of two flanking regions (δ and ϵ) with a cytoplasmic repressor (Baichwal *et al.*, 1992).

Recently, it was also shown that Pho4p is phosphorylated on the same tryptic peptides, *in vitro* and *in vivo*, by a Pho80p/Pho85p complex which could be immunoprecipitated from cell extracts (Kaffman *et al.*, 1994). Pho85p, which is 50%

homologous to the yeast CDC28 cyclin-dependent kinase, was shown to have kinase activity, but only in the presence of Pho80p (Kaffman *et al.*, 1994). Pho80p is 33% identical to HCS26 and other yeast cyclins, which are required by cyclin-dependent kinases for activity. The Pho80p and Pho85p proteins stably associate when expressed *in vitro*, and can phosphorylate *in vitro*-expressed Pho4p protein. In addition, Pho4p from cells carrying a *Pho80Δ* or *Pho85Δ* deletion appear to be hypo-phosphorylated. Recently, it was shown that Pho81p inhibited activity of the Pho80p/Pho85p complex when cells were grown in media depleted of phosphate (inducing conditions) (Schneider 1994). In addition, by coimmunoprecipitation Pho81p was shown to bind to Pho80p and to the Pho80p/Pho85p complex under all conditions, but not to Pho85p, and to inhibit Pho80p/Pho85p dependent phosphorylation of Pho4p *in vitro* and *in vivo* when overexpressed. The active inhibitor region of Pho81p, which alone could inhibit Pho80p/Pho85p activity *in vitro*, was localized to a 320 amino acid residue sequence which shows homology to $p16^{INK4}$, a putative tumor suppressor and potent inhibitor of the mammalian CDK4 kinase, (Schneider 1994). This region also contains six copies of the 33 amino acid residue ankyrin repeat motif believed to participate in protein-protein interaction (Schneider 1994). In summary, the current model for regulation of Pho4p activity suggests that in the presence of high phosphate media (repressing conditions) Pho81p is

bound to Pho80p/Pho85p, but is inactive, and the Pho80p/Pho85p kinase phosphorylates Pho4p thereby inactivating it and preventing transcription of the rAPase genes. In low phosphate conditions the bound Pho81p inhibitor inactivates Pho80p/Pho85p kinase, and the under-phosphorylated Pho4p activates transcription of the rAPase genes.

The STE12 activator. *STE12* encodes a 688 amino acid residue DNA-binding protein which mediates increased transcription of haploid-specific yeast genes involved in the mating response (Song *et al.*, 1991). In response to the mating pheromones, Ste12p activator protein binds to pheromone-response elements (PRE) upstream of the target genes and stimulates transcription of these genes. Induction of the expression of the target genes by Ste12p occurs in the absence of protein synthesis, suggesting that transcription activation is mediated by post-translational modification of existing Ste12p protein (Song *et al.*, 1991).

Deletion analysis has shown that the DNA-binding domain lies between residues 40 and 204, that deletions or random 6-residue insertions in this region affect DNA-binding, and that cells carrying these mutations are defective in mating (Kirkman-Correia *et al.*, 1993). In addition, it was shown that the transcription activation domain lies in the C-terminal region between residues 400-688. A double deletion of residues 1-214 and 473-688 from a LexA-Ste12p hybrid

resulted in constitutive expression of the reporter target gene. Deletion of either region alone resulted in a fully functional activator. This indicates that two negative regulatory domains exist within these two regions. Residues 253-305 and 305-406 contain functionally redundant, pheromone-responsive domains as deletion of either region, but not both, resulted in pheromone-responsive, functional activators (Kirkman-Correia *et al.*, 1993). These two regions are believed to be Ste12p targets of pheromone-induced modification. In response to pheromone stimulation, Ste12p protein undergoes hyperphosphorylation which correlates with the ability of the protein to activate transcription (Song *et al.*, 1991). The current model for regulation of Ste12p activity suggests that in the presence of pheromone the protein undergoes a conformational change which relieves inhibition or reveals a previously hidden transcription activation site. Pheromone induced phosphorylation of Ste12p is believed to play a role in this activating change.

The Put3 activator. *PUT3* encodes a 979 amino acid residue cysteine-rich, DNA-binding protein which binds to the upsteam promoter region of the genes involved in proline utilization (Marczal-DaCosta, 1990). Put3p, which is constitutively expressed and which binds under all conditions to its cognate UAS site is inactive in the absence of proline (Axelrod et al., 1991). However, in the presence of proline as the sole

nitrogen source, Put3p activates the expression of the target genes, indicating that the activity of the protein is regulated at a step subsequent to its binding to the UAS.

Put3p contains a cysteine-rich, DNA-binding domain between residues 30-60. *In vitro* DNA-binding studies showed that peptides containing the first 130 residues expressed in *E. coli* could bind DNA, and that deletion of residues 1-30 of Put3p did not affect binding (des Etages and Brandriss, personal communication). Residues 25-43 contain a putative nuclear targeting signal and residues 890-955, contain a transcription activation domain which, when fused to the Gal4p DNA-binding domain, activated transcription of a *GAL1-LacZ* reporter gene (des Etages and Brandriss, personal communication).

Seven constitutive point mutations have been isolated in the *PUT3* gene, four of which converted codon 956(Trp) to a nonsense codon and resulted in the deletion of the 23 C-terminal residues. This suggests a regulatory role for this region of the activator. However, deletion of the C-terminal 14 residues or the C-terminal 17 residues did not result in a constitutive activator (Marczak *et al.*, 1991). These mutant activators had wild type or near wild type activity under non-inducing conditions. The other three constitutive mutations, which converted residues 903(Leu) to Arg, 914(Asn) to Ile, and 683(Ser) to Phe, suggest that in addition to the C-terminal residues, other regions of the protein are involved in

regulating Put3p activity. Two non-inducible point mutations, which converted residue 409(Gly) to Asp, and 532(Gly) to Arg, occurred within a region of unknown function. Within this region, residues 453-537 show significant homology to regions in Gal4p, Leu3p and other yeast activators (Marczak *et al.*, 1991; des Etages and Brandriss, personal communication).

The rat glucocorticoid receptor (rGR). The rat glucocorticoid receptor belongs to a family of structurally related cytoplasmic, ligand-regulated transcription factors which, in response to hormone stimulation, bind to DNA elements (hormone response elements-HRE) upstream of the target genes and activate transcription (Godowski *et al.*, 1987; Evans, 1988). Deletion studies of the cloned genes have shown that the 777 amino acid receptor has a centrally located, cysteine-rich DNA-binding domain between residues 421-486 (Evans, 1988). A transcription activation domain is located in the region between residues 77 and 262 (Hollenberg and Evans, 1988). A second cryptic activation domain was mapped to a region between residues 526-556. The hormone-binding domain is located in the carboxy-terminal region between residues 532-697, adjacent to one of two nuclear localization signals (Godowski, *et al.*, 1987).

Regulation of the activity of the rGR is at the level of regulation of its subcellular location (Howard *et al.*, 1988). In the absence of hormone, a heat shock protein (Hsp90) binds

to and sequesters the receptor in the cytoplasm (Howard *et al.*, 1988). Binding of glucocorticoid hormone to the receptor releases bound Hsp90, and the receptor now localizes to the nucleus, where it can bind to HRE sequences and alter the transcription of its target genes (Picard *et al.*, 1988). When heterologous NLS sequences from SV40 large T-antigen are fused to rGR, the chimaeric gene localizes to the nucleus, but is unable to activate transcription (Picard *et a.*, 1988). This indicates that inhibition of nuclear localization is not the only level of regulation of rGR activity. Addition of hormone to cells carrying the chimaeric SV40/rGR gene results in induced transcription of the target genes, indicating that in addition to its function in nuclear localization, the hormone is also required for activation of receptor function (Picard *et al.*, 1988). Internal deletion of the hormone-binding domain resulted in constitutive expression of the target genes (Godowski *et al.*, 1987). This suggests that in hGR protein, the hormone-binding domain mediates a negative regulation on the activation function, and that binding by the hormone completely relieves this inhibition. The nature of this inhibition is not known, but conversion to an active form may involve a hormone-induced conformational change, and/or removal of a (non-HSP90) receptor-bound cytoplasmic inhibitor.

Maltose fermentation in Yeast.

Maltose fermentation in *Saccharomyces cerevisiae* requires two regulated enzyme activities, maltose permease and maltase. Both enzyme activities are induced by maltose and repressed by glucose in the medium. The product of a third gene encoding the MAL-activator is required for induction and in part, mediates glucose repression of the structural genes. Recessive uninducible mutations, and dominant constitutive mutations have been isolated in the activator gene. Constitutive mutations result in unregulated (maltose independent) expression of the maltose fermentative genes.

Yeast strains able to ferment maltose carry any one of 5 unlinked, structurally and functionally homologous *MAL* loci: *MAL1*, *MAL2*, *MAL3*, *MAL4*, and *MAL6* (Berge *et al.*, 1973; Needleman and Michels, 1983; Charron *et al.*, 1989). Three genes are encoded at each locus. *GENE1* encodes maltose permease, a membrane protein responsible for transporting the disaccharide across the plasma membrane (Cheng and Michels,1989); GENE2 encodes maltase, which cleaves maltose into its constituent glucose units, and GENE3 encodes the MAL-activator, a positive regulator of the system (Dubin *et al.*, 1985; Hong and Marmur, 1986; Chang *et al.*, 1988; Kim and Michels, 1988). At the *MAL6* locus, *MAL61* encodes the maltose permease, *MAL62* encodes maltase, and *MAL63* encodes the MAL-activator. The sequence of these genes and their predicted amino acid sequences have been reported (Cheng and Michels,

1989; Hong and Marmur, 1986; Kim and Michels, 1988). *Saccharomyces* strains carrying any one of the complete *MAL* loci will ferment maltose sugar. Naturally occurring variant strains have been described which carry copies of the structural genes for maltose permease and maltase, but which lack all functional copies of the activator gene (Needleman and Michels, 1983; Naumov *et al.*, 1994). Such strains do not ferment maltose, and do not induce the expression of the structural genes in the presence or absence of maltose (Needleman and Michels, 1983). Similarly, *MAL* strains which carry point-mutations or deletion/disruptions of their activator gene no longer ferment maltose (Chang *et al.*, 1988). This non-fermenting phenotype can be completely rescued by a plasmid carrying a copy of the MAL-activator gene. Thus, the activator gene encodes a positive regulator which is required in order for yeast cells to induce the expression of the structural genes of the *MAL* system in the presence of the inducer, maltose (Chang *et al.*, 1988).

The MAL-Activator

The *MAL63* gene which encodes the activator at the *MAL6* locus was cloned and sequenced (Kim and Michels, 1988; Solliti and Marmur, 1988). The predicted amino acid sequence suggested that *MAL63* encoded a 470 amino acid residue protein with a cysteine-rich amino-terminal domain (residues 8-38) homologous to the cysteine- rich, zinc-dependent DNA-binding domains characterized in Gal4p and other yeast activators. A putative

nuclear localization signal is located in the N-terminal region between residues 40-55 (Rihs *et al.*, 1991). Two acidic regions are located between amino acid residues 104 and 116, and residues 133 and 149 in the amino terminal half of the protein. In the C-terminal half of the activator, three additional acidic regions are found between residues 287 and 301, residues 309 and 326, and residues 450 and 470. Highly acidic regions, also capable of forming α-helices, had previously been shown in Gal4p and other yeast activators, to be the domains responsible for transcription activation (Johnston, 1987), and it is suggested that these regions somehow make contact with the RNA polymerase II-dependent transcription complex at the promoter region. However, recent evidence which shows that the acidic character of this region in Gal4p is unimportant for transcription, and that the region predominantly forms a β-sheet, have left open the question as to what constitutes an activation domain (Leuther *et al.*, 1994; Van Hoy *et al.*, 1993).

Mal63p is a DNA-binding protein. The *MAL* structural genes, *MAL61* and *MAL62*, are coordinately and divergently transcribed from a common 874 basepair promoter region (Needleman *et al.*, 1984; Levine *et al.*, 1992). Deletion analysis of the bidirectional promoter defined a 68 basepair region sufficient for maltose-dependent and Mal63p-dependent regulation of the structural genes (Levine, *et al.*, 1992). This sequence is referred to as the UAS_{MAL}. Kim (1992) using

the gel mobility shift assay, showed that (*E. coli* expressed) residues 1-111 containing the postulated DNA-binding domain of the Mal63p protein retarded the electrophoretic mobility of, and therefore bound to, a 40 basepair double stranded oligonucleotide whose sequence was derived from that of the UAS_{MAL}. In addition, Kim showed that independent mutations in three of the six cysteine residues within the proposed DNA-binding zinc cluster (residues 8-38) abolished DNA binding *in vitro*, and that the mutant activators were unable to activate maltase gene expression *in vivo*. Ni and Needleman (1990), using the DNA footprinting technique, demonstrated that Mal63p protein (produced in *E. coli*) protected two sites in the *MAL61-MAL62* intergenic region. Site 1 coincided with the 40 basepair sequence used by Kim (1992). Site 2 mapped outside of the UAS_{MAL} defined by Levine *et al.* (1992). Thus, the Mal63p activator appears to mediate induction of the expression of the two structural genes by binding at the UAS_{MAL} and making contact directly or indirectly with the transcription machinery.

Two classes of mutations have been isolated in the MAL-activator; recessive uninducible mutations, which result in nonfermenters, and dominant constitutive mutations which express the structural genes even in the absence of the inducer, maltose (ten Berge *et al.*,1974; Zimmerman and Eaton, 1974; Khan and Eaton, 1971). Sequencing of six independent uninducible *mal63* mutations revealed that three resulted in

nonsense mutations in codons 320 and 333 (J. Kim, unpublished results). Of the other three mutant alleles, one carried two missense mutations converting Gln85 to Pro, and Ala119 to Val. Two others each contained single mutations converting Gly148 to Glu, and Ala254 to Thr, respectively (J. Kim, unpublished results). A temperature sensitive revertant of the allele carrying the Pro85, Val119 mutations had two additional alterations, one in Cys86 (to Tyr) and the other in Asp250 (to Asn). These mutations suggest that the N-terminal region of Mal63p, outside of the DNA-binding domain, contains required functional domains that can be inactivated by single point mutations. In addition, residue 254 also appears to be important for function.

Two classes of constitutive, *MAL*-activator mutations have been identified. Eaton and Khan (1971) described a dominant constitutive strain, 1403-7A, which carried the *MAL4* locus and expressed high levels of maltase even in the presence of glucose. The *MAL4* locus was cloned from this strain and the activator gene, *MAL43*, was shown to convert a nonfermenting strain lacking an activator to a dominant constitutive, glucose-repression-insensitive phenotype (Charron and Michels, 1988). Therefore, *MAL43* contained the genetic alteration responsible for its dominant mutant phenotype.

Five dominant constitutive mutations also have been isolated in *MAL2* strains (Zimmerman and Eaton, 1974). Two of these are sensitive to glucose repression, while the other

three show partial insensitivity to glucose repression. Charron and Michels (1987) preliminary results suggest that one of these *MAL2-C* mutations maps to *MAL23*.

Maltose fermenting revertants of uninducible *mal6* strains were isolated by ten Berge (1973) and were found to express the structural genes constitutively. The mutations underlying the constitutive phenotype were mapped to the *MAL6* locus to a tandem, upstream imperfect copy of the *MAL63* activator gene, called *MAL64*. The wild type *MAL64* gene and two of the constitutive mutant alleles (*MAL64-C2, MAL64-R10*) were cloned and sequenced by L. Wojciechowicz (Ph.D. thesis, 1993).

While the wild type nonfunctional *mal64* gene was unable to complement *mal63Δ* strains, the mutant *MAL64* genes were found to be dominant to the wild type *MAL63*, and partially insensitive to glucose repression. The DNA sequence of *mal64* showed that it encoded a predicted 470 amino acid residue protein which is 85% homologous to *MAL63*. *MAL64-C2* and *MAL64-R10* however, encode C-terminally truncated proteins. *MAL64-C2* has a premature termination codon at position 306, while *MAL64-R10* has a termination codon at position 282. This finding suggests that these mutations result in the removal of a C-terminal regulatory region which inhibits function of the activator protein in the absence of inducer. Experiments with hybrid gene fusions between *MAL63* and *MAL64* support this hypothesis. A *MAL64/63* hybrid gene encoding codons 1-215 of *MAL64* and codons 216-470 of *MAL63* expressed from the *mal64*

promoter was found to be maltose inducible.

The goal of my thesis work is to define the functional domains of the MAL-activator. To this end, I carried out an analysis of the Mal63p activator using the *LexA*-fusion methods developed by Hanes and Brent (1989). A molecular genetic analysis of the constitutive Mal43-Cp activator was undertaken in order to understand the genetic basis of its mutant phenotype. Finally, the two hybrid system was used in an effort to identify interactions within and between Mal63 protein molecules.

Materials and Methods.

Yeast strains. The following strains were used in this study: Strain B16 (*MATa MAL23 MAL21 MAL22 mal11 MAL12 mal13 ura3-52 his3Δ200 ade1*) was isolated from a cross between strains SEY6210 (*MATα mal13Δ AGT1 MAL12 mal33Δ MAL31 MAL32 ura3-52 his3Δ200 leu2-3,112, trp1-Δ901 lys2-801 suc2-Δ9*) and MCY101-3A (*MATa MAL21 MAL22 MAL23 mal11 MAL12 mal13Δ ura3-52 leu2-3,112 ade1*) (Evan Reid, personal communication). Strain 340-2A (*MATα mal13Δ AGT1 MAL12 ura3-52 ade*) was used as a MAL-activator deficient tester strain and is described by Charron and Michels (1988). Strain YPH500 (*MATa AGT1 MAL12 mal13Δ MAL31 MAL32 mal33Δ ura3-52 his3Δ200 leu2-3,112 trp1-Δ901 lys2-801 ade2*) was used as a tester strain for *LacZ* reporter gene expression studies and is described by Sikorsky and Heiter (1989).

Bacterial growth and transformation. *E. coli* strains RR1 and DH5α were used for plasmid amplification. Cells were grown at 37°C in LB or YT broth. Antibiotics were added to force cells to maintain plasmids as described (Maniatis *et al.*, 1982). *E. coli* recA$^-$ strains JM109 and MV1190 were used to amplify M13 phage containing recombinant DNA constructs. These strains were maintained on M9 minimal media plates and grown in LB

broth (Maniatis *et al.*,1982). *E. coli* cells were made competent for plasmid transformation using the calcium chloride method of Hanahan (1983) and stored as described for up to 6 months. Transformants were selected as antibiotic resistant colonies on LB plates containing antibiotics. To locate those colonies carrying the desired recombinant plasmids, transformants were screened by colony hybridization using ^{32}P-dCTP (Amersham) labeled DNA probes, or by the polymerase chain reaction (PCR) using insert-specific oligonucleotide primers. Recombinant plasmid DNA was then isolated from the positive colonies and the insert characterized.

Yeast growth and transformation. Yeast cells were grown at 30°C on YPD (1% yeast extract, 2% peptone, 2% dextrose) or synthetic minimal media (SM)(Bio 101 Inc.) containing appropriate carbon sources but lacking the amino acids necessary for plasmid retention by cells (Sherman *et al.*, 1983). Yeast cells were made competent for transformation and subsequently tranformed using the lithium acetate method (Ito *et al.*, 1983) or with modifications (Hill *et al.*, 1991). Transformants were selected on synthetic minimal media (SM) lacking appropriate amino acids and then screened for episomal plasmid segregation by first growing under non-selective conditions (YPD) and then screening under selective conditions (SM) for the presence or absence of the plasmid borne

nutritional marker.

Recombinant DNA techniques. DNA preparations, digestions and ligations were carried out according to standard protocols (Ausubel, *et al.*, 1989, and Maniatis, *et al.*, 1982). Commercially available kits were used for several procedures in this work. Plasmid DNA was purified from bacterial cultures using Qiagen DNA purification kits. DNA probes for hybridization were labeled using the Random Priming DNA labeling kit (BioRad) or the Megaprime labeling kit and ^{32}P-dCTP from Amersham. DNA fragments for ligation were extracted from agarose gels using the Geneclean kit (Bio 101 Inc.). *In vitro*, oligonucleotide-directed mutagenesis was carried out using the BioRad Muta-Gene kit and instructions provided by the manufacturer. For DNA sequencing of M13 clones single-stranded DNA was prepared according to protocols provided by Amersham. Sequencing reactions were performed by the dideoxy method (Sanger *et al.*, 1977) using reagents purchased from United States Biochemical (USB Version 2.0 Sequenase kit) and ^{35}S-dATP (Amersham). Oligonucleotide primers used in mutagenesis, PCR and sequencing reactions were made and purified using the Applied Biosystems Inc. DNA synthesizer Model 381A, and reagents purchased from ABI. In order to generate a continuous DNA sequence overlapping DNA fragments were located using the Genetics Computer Group Sequence Analysis Software (Devereux *et al.*, 1984).

Table 1. Oligonucleotides used in sequencing, mutagenesis and PCR-ampliification. Oligonucleotides sequences are written in 5′-3′ direction. Numbers given represent primer annealing positions in the open reading frame (ORF) of *MAL63*. Where appropriate, altered sequences or restriction endonuclease sites within mutagenic oligonucleotides are underlined.

[a] Basepair +1 is the first base of the open reading frame (ORF).

[b] K4 shown to anneal to another undefined site in the coding strand of *MAL63* (J. Kim, personal communication).

[c] NC indicates that primer anneals to non-coding strand.

[d] C indicates that primer anneals to coding strand.

Table 1. Oligonucleotides used in sequencing, mutagenesis and PCR-amplification.

Oligonucleotide	Sequence(5'-3')	Position in *MAL63* (basepairs in ORF[a])	Comments
A1	TTCGGTGAACAACAACTC	657-672	
A2	CGCTGCATTCAGCGCAAT	78-93	
A3	TTCACAAGCTTCTAGAAG	317-334	
A4	AAAGAAAATTCATCCGC	1062-1044	
A5	GATAACTTATATGTAATT	276-291	
A6	TAGCCAATGCATTGGTAGATG	1185-1206	
A8	GTCAATACGTGAGAAC	448-464	
K2	TGAGTTGTTGTTCACCGA	675-657	
K4	TAAAAAACACTTTCCTGGTAT	858-840	Anneals to both strands[b]
K5	TCATATGAAGTACTAGTTT	1082-1063	
K6	TCATACTTATTTACGACAGC	321-303	
K7	GTATAAGTGAACGGCGTGAA	1418-1398	Anneals 8 bp 3'of TAA codon
K8	GCAAAGAGTGCACAACTG	465-450	
K9	CGAATCATGACATACTAT	516-531	
K11	CGGAAGGTTCTTACATAC	222-237	
K12	TAGCTTCTTACACCCTCG	1488-1470	Anneals 78 bp 3'of TAA codon
K13	TCCATTAGAGCAGGAAGCT	141-159	
L6	GCGGATGAATTTTCTTT	1044-1062	

Mutagenic oligonucleotides(altered sequence underlined)

A18	CCGTATCCTTTTTAGAGAGTCAGATCTTAGGCTCGTGACACACTT	Deleted codons 250-300. Inserted *Bgl*II site.
A19	AGTATTCGAAAGAAAATTAGATCTTTCGGTGCAGGAATCATC	Deleted codons 301-350. Inserted *Bgl*II site.
A20	ATACTTATTTACGACATCAGATCTCATCCGCATACCTTTCAT	Deleted codons 351-400.

Table 1. (continued)

Oligonucleotide	Sequence(5′-3′)	Comments
A21	CAAAGGTTTAGAAATGGGAGATCTTACCAATGCATTGGCTAT	Inserted *Bgl*II site. Deleted codons 401-450. Inserted *Bgl*II site.
A22	TATATGTGTGGCATTAGTATTCGAAAAAAAATTCATCCGCATATC TTTCTTATTAAGTACTAG	Codons 344,345,347,353,358 changed to *MAL43-C* sequence.
A23	ACCATGTACATCATACAGGTCGATCGGAGTTAAAAGCGTGTCCTG CAACATGTCCTTAGCAATTTCGACTGGTAT	Codons 367,371,374,378,379 changed to *MAL43-C* sequence.
A24	GTCTTCATCTTTGGAATCATCATTTAAGCGCAAAGGTCTAGAAAT GGGCAGATCAATTAGGGCACTTTGACATTTCGTTGAAAA	Codons 445,449,454,457,461 changed to *MAL43-C* sequence.
A25	TCACTTGATATAGAGCCATGGTCTTACGGATACATAGACTTTCTC TTTTCTCGGCACT	*MAL43-C* codons 320, 327 changed to *MAL63* sequence.
A26	GCATTGGTAGACGTCGTAAATAAGTATGATCACAATATG	*MAL43-C* codon 404 changed to *MAL63* sequence.

PCR primers (Restriction sites underlined)

A10	CGGGATCCTTCTTCAACCGTTGAAAAAGAGA	*Bam*HI site 5′ of codon 37(NC[c])
A11	CGGGATCCCAATGCTTTCCTACGATGATCTT	*Bam*HI site 5′ of codon 99(NC)
A12	CGGGATCCAGCAGTTGTGCACTCTTTGCATG	*Bam*HI site 5′ of codon 150(NC)
A13	GGGAATATTCAACGGTTGAAGATAAGT	*Sst*I site 3′ of codon 40(C[d])
A14	CGGGATCCCCATCTGCACTTCCGCTAT	*Bam*HI site 3′ of codon 61(C)
A15	CGGGATCCCTGGCCAATTTACATATAAGTT	*Bam*HI site 3′ of codon 99(C)
A16	GCGGATCCTTCGGTGCAGGAATCATCGAC	*Bam*HI site 3′ of codon 300(C)
A17	CGGGATCCGCCGTGGGCCTCATCAAGATA	*Bam*HI site 5′ of codon 199(NC)
A27	CGGGATCCTTACTTTCCTGGTATAGTGAA	*Bam*HI site 5′ of termination codon at position 284(NC)
A28	CGGAATTCATGGGTATTGCGAAACAGTCT	*Eco*RI site 5′ of codon 1(NC)
A29	GCGGATCCCACACTCTATCAGTATATCTATC	*Bam*HI site at +1464(NC)
A30	GCGGATCCATGGGTATTGCGAAACAGTCT	*Bam*HI site 5′ of codon 1(NC)
A31	CGGGATCCACCCCGTGCTGCCTGCCACTT	*Bam*HI site 5′of -154(NC)
A32	GCGAATTCATAGCCAATCCATTGGTA	*Eco*RI site 5′ of codon 395(NC).

Table 2. Plasmids used in this work.

Plasmid	Description	Source and/or Reference
YCp50	*URA3* CEN4 ARS6 (1-2 copies per yeast cell)	Rose *et al.*, 1987
YEp352	*ARS6 URA3* (40 copies per yeast cell)	Hill *et al.*, 1986.
pSH2-1	*HIS3*; contains codons 1-87 of *LexA*	Hanes *et al.*, 1989
pBTM116	*TRP1*; contains codons 1-202 of *LexA*	Ruden *et al.*, 1991
pSH18-18	*URA3*; contains *LacZ* reporter with 6 *LexA* binding sites	Brent *et al.*, 1987
pGBT9	*TRP1*; contains codons 1-147 (DNA-binding domain) of *GAL4*	Bartel *et al.*, 1993.
pGAD.1R	*LEU2*; contains codons 768-881 (transcription activation domain) of *GAL4*	Bartel *et al.*, 1993.
pRS315	*LEU2* CEN4 ARS6	Sikorski and Heiter, 1989.
pMAL23	Rescue plasmid containing *MAL23*	This work
pM43B8	Rescue plasmid containing *MAL43-C*	Charron and Michels, 1987.

Cloning of *MAL23* by plasmid rescue. The integrating plasmid pA42 which carries the bacterial ampicillin resistance gene, the yeast *LEU2* gene, and a 2.6 kb *Bam*HI/*Bgl*II fragment from the upstream untranslated region of *MAL23* was targeted for integration at the *MAL2* locus in the region upstream of *MAL23* by digesting with *Hind*III and transforming yeast strain B16. Integration at the correct locus position was confirmed by Southern analysis of restriction endonuclease digested genomic DNA from several transformants and by crosses to a non-fermenting strain to show linkage of the plasmid *LEU2* marker to *MAL2*. The construction of this strain called D72-1 was carried out by Evan Reid in our laboratory. To rescue *MAL23*, 35μg of genomic DNA from a single copy integrant was digested with *Sal*I and allowed to self-ligate. The ligated DNA was then transformed into *E. coli* strain RRI. Two ampicillin resistant transformants carrying a 10.5Kb rescue plasmid were isolated and one, pMAL23#1, was further characterized. The restriction map of the yeast insert is consistent with that reported for *MAL23* and flanking sequences by Charron *et al.* (1989) and the plasmid complemented the MAL-activator deletion strain 340-2A making it a maltose fermenter.

Sequencing *MAL23* and *MAL43*-C. A 3.0 kb *Kpn*I fragment containing *MAL23* was subcloned from the pMAL23 rescue plasmid into M13 phage in both orientations. Both strands of the gene were completely sequenced. Similarly, a 3.0 Kb *Kpn*I fragment

containing *MAL43*-C was subcloned from plasmid pM43BS (Charron and Michels, 1988) into M13 phage in both orientations and both strands sequenced. Oligonucleotide primers used in sequencing reactions are listed in Table 1.

Construction of *MAL63/43*-C hybrid molecules. To simplify the construction of *MAL63/43*-C hybrid genes plasmid YCpMAL63ΔRI (Lori Wojciechowicz, 1993) was used. This construct contains a *MAL63* allele in which the 5′ *Eco*RI restriction site had been deleted from the upstream untranscribed region of the gene. Since the *Eco*RI site at codons 215/216 and the downstream *Eco*RI site remain intact, digestion with *Eco*RI releases the 3′ half of *MAL63* containing codons 215 to 470 allowing this fragment to be replaced by a corresponding mutated *Eco*RI fragment. Plasmid YCpMAL63ΔRI was digested with *Eco*RI and allowed to self ligate to produce plasmid YCpMAL63Δ3′RI. This was used as a recipient in the following constructions.

YCpMAL63(1-215)/43-C(215-470) was constructed by ligating a 1.1 kb *Eco*RI fragment (from *MAL43-C*) containing codons 215-470 and 3′ downstream sequences of into YCpMAL63Δ3′RI. Because of difficulties experienced in amplifying this construction in *E. coli*, the ligation product was transformed directly into the yeast strain 340-2A. Ura+ transformants were selected and tested for the ability to ferment maltose. Using PCR and *MAL63* derived primers, MAL-activator gene sequences were amplified and the construction confirmed by

restriction digestion analysis of the PCR product.

To create YCpMAL63/43-C(341-361), YCpMAL63/43-C(361-385) and YCpMAL63/43-C(439-466) the *Eco*RI fragment from *MAL63* containing the 3′ end of the gene was cloned into M13 (Lori Wojciechowicz, 1993. Ph.D. thesis) and mutagenized by oligonucleotide-directed mutagenesis, using protocols provided by BioRad. Three mutagenic oligonucleotides, A22, A23, A24, were synthesized to replace the *MAL63* codons 341-361, 361-385, and 439-466, respectively, with the corresponding codons from *MAL43-C*. The sequences of these oligonucleotides are listed in Table 1. The mutagenized *Eco*RI fragments were then inserted into YCpMAL63Δ3′RI and the insert orientation confirmed by restriction digestion analysis. To create the double mutation, YCpMAL63/43-C(361-381)(439-466), DNA from a clone carrying a sequence-confirmed mutant hybrid, *MAL63/43-C*(361-385), was used as the template in a second mutagenic reaction as described above.

Construction of *MAL43*-C point mutations. YCpMAL43-C(Y320W,Y327S) was constructed by site-directed mutagenesis of the *MAL43-C* sequence using a mutagenic oligonucleotide, A25. Codons 320 and 327 of *MAL43-C* (both of which encode tyrosine) were converted to the corresponding residues of *MAL63* (tryptophan and serine respectively). A sequence confirmed clone from this mutagenic reaction was then used as the template in a second mutagenic reaction to create YCpMAL43

(Y320W,Y320S,S404N). In this reaction, oligonucleotide A26 was used to convert codon 404 (encoding serine in *MAL43-C*) to the asparagine residue found in *MAL63*. This mutation was confirmed by DNA sequencing. To subclone the mutagenized genes from the M13mp18 construct into YCp50 two oligonucleotide primers, A31 and A29, both of which carried *Bam*H1 sites, were used to PCR-amplify a 1.484kb fragment containing the entire coding region and some upstream and downstream sequences. The PCR product was ligated into the *Bam*HI site of YCp50.

Construction of *LexA-MAL63* hybrids. pLexA-MAL63(2-470), pLexA-MAL63(2-456), and pLexA-MAL63(215-470) were constructed by L. A. Wojciechowicz (1993). pLexA-MAL63(2-216) was created by deleting the *Eco*RI fragment containing codons 216-470 and 3' downstream sequences from pLexA-MAL63(2-470). This fuses codon 216 to vector sequences and results in the C-terminal addition of 24 amino acid residues (FPGIRRPAAKLIPGEFLMIYDFYY) before an in-frame termination codon is reached.

pLexA-MAL63(Δ250-300), pLexA-MAL63(Δ301-350), pLexA-MAL63(Δ351-400) and pLexA-MAL63(Δ401-450) were created in a two step process. Oligonucleotides A18, A19, A20, and A21 were used to delete codons 250-300,301-350, 351-400 and 401-450, respectively, from the *Eco*RI fragment of *MAL63* containing codons 216-470 and 3' downstream sequences using site directed

mutagenesis. The alterations were confirmed by completely sequencing the *Eco*RI fragments and the mutagenized *Eco*RI fragments were then cloned into LexA-MAL63(2-216) in the correct orientation.

pLexA-MAL63(1-283) was created as follows. Two oligonucleotide primers, A27, which has a 5′ *Eco*RI site and A30, which carries a 5′ *Bam*HI site were used to amplify a 0.864kb fragment containing codons 1-283 of *MAL63*. Primer A30 also has a termination codon at the position of codon 284. Since this 0.864kb fragment contains an internal *Eco*RI site at codons 215/216, digestion of the PCR product resulted in two fragments containing codons 1-216(*Eco*RI/*Eco*RI) and codons 215-283(*Eco*RI/*Bam*HI). These were ligated to *Bam*HI/*Eco*RI digested pSH2-1. The structure of this three fragment ligation product was confirmed by PCR and restriction digestion. pLexA-MAL63(215-283) was created by digesting pLexA-MAL63(1-283) with *Eco*RI, releasing a 0.653kb fragment containing codons 1-215, and allowing to self-ligate. This resulted in the in-frame fusion of codons 215-283 to the LexA DNA-binding domain encoded by the vector.

pLexA-MAL63(37-470), pLexA-MAL63(99-470), pLexA-MAL63(150-470) and pLexA-MAL63(395-470) were constructed as follows. Three mutagenic oligonucleotides, A10, A11, A12, each carrying a *Bam*HI site 5′ of codon 1, were used as upstream primers to PCR-amplify DNA fragments carrying *MAL63* codons 37-470, 99-470, 150-470, respectively. To maintain the

correct reading between the *MAL63* fragment and *LexA*(1-87), two bases were inserted immediatedly 3'to the *Bam*HI site in each of the four primers. The downstream primer, A29, also contained a 5' *Bam*HI site and was used in all three PCR reactions. PCR products were ligated into the *Bam*HI site of pSH2-1.

pLexA-MAL63Δ41-66 was created from pLexA-MAL63(37-470) as follows. A 0.136 kb fragment containing codons 1-40 of *MAL63* was PCR amplified using oligonucleotide primer A30, which contains an *Eco*RI site and anneals at codon 1, and primer A13, which contains a 5' *Sst*I site and anneals at codon 40. This, along with a 1.2 kb *Sst*I/*Bam*HI fragment from pLexA-MAL63(37-470) containing *MAL63* codons 66 to 470, was ligated to *Eco*RI/*Bam*HI digested pSH2-1. pLexA-MAL63Δ62-98 was created from pLexA-MAL63(99-470) by a similar three fragment ligation. A 0.199kb DNA fragment containing codons 1-61 of *MAL63* was PCR-amplified using oligonucleotide primers A30 (described above) and A14, which anneals at codon 61 and contains a 5' *Bam*HI site. This was ligated to a 1.2 kb *Bam*HI fragment from pLexA-MAL63(99-470) containing codons 99-470 and pSH2-1 (*Eco*RI/*Bam*HI digested).

pLexA-MAL63(Δ100-149) was created by amplifying a 0.3 kb fragment containing codons 1-99 of *MAL63* using the oligonucleotides A30 and A15, which contains a 5' *Bam*HI site. This fragment was ligated to a 1.0 kb *Bam*HI fragment from pLexA-MAL63(150-470) containing codons 150-470 and pSH2-1

(*Eco*RI/*Bam*HI digested).

To create pLexA-MAL63(395-470) primer A32, which has a 5'*Eco*RI site, and primer A29 were used to PCR-amplify a 0.4 kb *MAL63* DNA fragment containing codons 395-470, which was ligated into *Eco*RI/*Bam*HI digested pSH2-1. The orientation of all inserts was verified by restriction digestion analysis.

pLexA-MAL43-C(215-470) was created by cloning a 1.1 kb *Eco*RI fragment from *MAL43-C* containing codons 215-470 plus 3' downstream sequences into the *Eco*RI site of pSH2-1. The correct orientation of the insert was verified by restriction digestion.

Construction of pGAD-MAL63 hybrids. To construct pGAD-MAL63(1-300), a 0.9 kb DNA fragment containing codons 1-300 of *MAL63* was amplified by PCR using oligonucleotide primers A29, which anneals at codon 1 and A16, which anneals at codon 300. Both primers contain 5'*Bam*HI restriction sites. The PCR product was cloned into the *Bam*HI site of pGAD.1R (obtained from Stan Fields; Bartel *et al.*, 1993) which contains codons 768-881 of *GAL4* and encodes only the transcription activation domain. Similarly, pGAD-MAL63(199-470) was constructed by amplifying codons 199-470 of *MAL63* on a 0.9 kb fragment using primers A17 and A29 and cloning into pGAD.IR. pGAD-MAL43-C(199-470) was also constructed using primers (A17 and A29) to clone a PCR-amplified 0.9 kb fragment, containing codons 199-470 of *MAL43-C*, into pGAD.IR.

Protein extract preparation. Protein extracts were prepared as outlined in Mylin *et al.*, (1989). Cells were harvested from overnight cultures at OD_{600nm}= 0.3-0.7. After chilling on ice for 10 minutes cells were washed once with cold water, once with breaking buffer (50mM $NaPO_4$ pH 7.2, 5mM EDTA), and resuspended in 0.3ml breaking buffer containing protease inhibitors and a reducing agent (1mM PMSF, 0.2 mM Na_3VO_4, 2μM pepstatin, 0.6μM leupeptin, 20 μg/ml aprotinin, 1mM DTT). An equal volume of 0.45mm glass beads (Sigma) was added and cells were broken by four 30 second pulses of vortexing separated by 30 second rests on ice. Extracts were placed in 1.5 ml microfuge tubes and the beads washed with 0.3ml of breaking buffer. The wash was combined with the extract in the microfuge tube, 0.2 volumes of 5X electrophoresis sample buffer(5% SDS, 50% sucrose, 50 mM Tris-Cl pH 8.0, 5mM EDTA, 0.16M DTT) was added and the mixture boiled for 5 minutes. The extracts were then centrifuged for 5 minutes at room temperature, and the supernate removed and stored at -70°C. Protein concentration in crude extracts was determined using the BioRad Protein Determination DC kit which is compatible with the high concentrations of SDS used in the sample buffer during the preparation of crude cell extracts.

Western blot analysis. SDS-PAGE gels were prepared accoding to the protocols given in Ausubel *et al.*(1989). 20-50 μg of crude protein extract was loaded onto polyacrylamide gels and

electrophoresed at 40mA. Separated proteins were transferred to nitrocellulose membranes for 1 hour at 80 volts in the recommended transfer buffer (22.33g Glycine, 4.83g Tris base, 1.6 liters deionized water, 400 mls methanol). The blots were blocked overnight at 4°C in PBS (5mM Na_3PO_4 pH7.2, 137mM NaCl) with 5% Carnation nonfat dry milk, 3% gelatin, 3% BSA, 0.1% Tween-20. Blots were probed using rabbit anti-LexA antibodies (obtained from Roger Brent) followed by alkaline phosphatase conjugated, goat anti-rabbit antibodies (Sigma). Protein bands were visualized using the Enhanced Chemiluminescence technique (ECL-kit, Amersham).

Enzyme assays. β-galactosidase activity was assayed according to protocols by Ausubel *et al.*(1989) using crude cell extracts. Specific activity is expressed as nmoles ONPG liberated per minute per mg protein. Maltase activity was assayed as outlined by Dubin *et al.* (1985). Specific activity is expressed as nmoles PNPG/minute/mg protein.

RESULTS

Molecular analysis of maltose induction and a constitutive MAL-activator gene.

A. Domain analysis of the MAL63 activator.

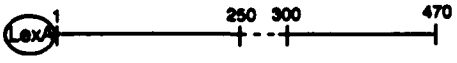
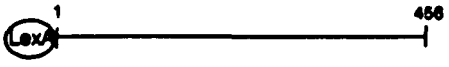
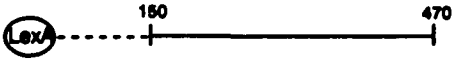
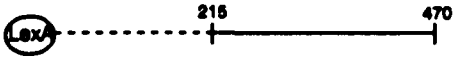
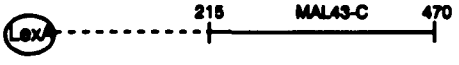
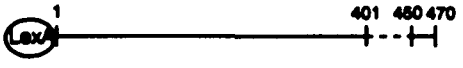
To identify functional regions of *MAL63* we carried out a deletion mutational analysis of the gene. As a transcription activator, Mal63p is expected to contain regions responsible for nuclear targeting, DNA-binding, inducer-responsiveness and transcription activation. Previously, it had been determined that Mal63p is a positive regulator of the *MAL*-structural genes (Chang *et al.*, 1988; Dubin *et al.*, 1986; Kim and Michels, 1988), and that residues 8-38 contained a cysteine-rich, zinc cluster region required for binding to the UAS_{MAL} located upstream of the *MAL*-structural genes, *MAL61* and *MAL62* (Kim, 1991; Ni and Needleman, 1989; Levine *et al.*, 1990;). However, other regions of the Mal63p protein required for its function as a *trans*-activator remained unidentified. To identify other functional domains of Mal63p we used site-directed mutagenesis to create N-terminal, C-terminal and internal deletions and tested their function by fusing the mutant alleles, in-frame, to the region encoding the DNA-binding domain (codons 1-87) of the bacterial gene, *LexA* (Hanes and Brent, 1989). The ability of the hybrid proteins

to activate expression of a *LacZ* reporter gene, under the control of six LexAp binding sites, was assayed in yeast strain YPH500 which also contained an episomal wildtype *MAL63* gene (to insure the availability of the inducer, maltose). β-Galactosidase assays were performed on cultures grown under uninduced (glycerol/lactate) and induced (maltose) conditions (Table 3). Those hybrids shown to activate reporter gene expression were tested for their ability to activate expression of the *MAL* structural genes as follows. The plasmid carrying *MAL63* was cured from the strain and the ability to ferment maltose determined (Table 3). Maltase assays were performed on strains shown to ferment maltose and/or activate reporter gene expression. Finally, using anti-LexA specific antibody, the expression of stable LexA-Mal63 fusion proteins was confirmed by western analysis of protein extracts from cells grown in maltose and glycerol/lactate.

The hybrid protein, LexA-Mal63(2-470)p, containing the full length Mal63p fused to LexA(1-87)p is a bifunctional activator that stimulates maltase gene expression and activates expression of the *LacZ* reporter gene. Expression of *LacZ* is induced approximately 100-fold by maltose while maltase levels are induced only approximately 20-fold. Deletion of the N-terminal 37 residues, which contain the DNA-binding zinc cluster region, resulted in a functional maltose-inducible activator of *LacZ*. In the presence of maltose,

Table 3. Activation of β-galactosidase and maltase expression by *LexA-MAL63* hybrid genes.

Fusion plasmids were constructed as described in Materials and Methods and transformed into strain YPH500 carrying the episomal *LacZ* reporter gene construct pSH18-18. Cells were grown in synthetic (SM) media lacking uracil, histidine and leucine but containing 3% (vol/vol) glycerol-2% (vol/vol) lactate (Gly/Lac), Gly/Lac + 2% (wt/vol) maltose as noninducing and inducing carbon sources, respectively. β-Galactosidase specific activity is expressed as nmoles o-nitrophenyl-β-D-galactopyranoside (ONPG) liberated per minute per mg protein at 28°C. Maltase activity is expressed as nmoles of p-nitrophenyl-α-D-glucopyranoside (PNPG) split per minute per milligram of protein at 30°C. Values given are the average of two or three trials. Errors were typically less than 30%.

	β-Galactosidase Activity		Maltose Fermentation	Maltase Activity	
	Maltose	Gly/Lac		Maltose	Gly/Lac
	1,847	15	++	2338	111
	1,279	12	-	136	19
	16,652	9	-	88	21
	21,402	12	-	92	20
	10	14	ND	ND	ND
	3,831	14,421	++	863	1331
	34	17	ND	ND	ND
	11	14	ND	ND	ND
	582	140	-	105	21
	3	20	ND	ND	ND
	7	15	ND	ND	ND
	2	14	ND	ND	ND
	7	10	ND	ND	ND
	3	7	ND	ND	ND
	3	11	ND	ND	ND
	3	14	ND	ND	ND
	19	16	-	92	25

cells carrying this hybrid gave β-galactosidase activity levels comparable to the wildtype hybrid. However, this strain could not ferment maltose nor induce maltase expression. This result is consistent with the fact that this hybrid activator is missing a region previously shown to be important for DNA-binding to the UAS_{MAL}. Nevertheless, it is important to note that it is fully functional as an inducible transcription activator.

Strains expressing the hybrid proteins LexA-Mal63Δ40-66p and LexA-Mal63Δ60-99p exhibited over 1,000-fold maltose-induced expression of β-galactosidase activity. This is 8- to 10-fold higher than the induced expression levels seen in strains expressing the full-length *LexA-MAL63* fusion but neither strain could ferment maltose. This result indicates that residues 1-100 are not required for maltose-inducible transcription activation, and that, in addition to the zinc cluster (residues 8-38), the immediately adjacent region (residues 40-99) is required for the MAL-activator to bind to the UAS_{MAL}. Additionally, this region appears to inhibit reporter gene expression.

The hybrid protein carrying a deletion of residues 100-149 was non-functional. Cells expressing this protein gave background levels of *LacZ* expression indicating that the deleted residues were required for activator function. Unfortunately, in transformants carrying the hybrid construction, *LexA-MAL63(99-470)*, the hybrid activator

protein could not be detected in Western blot analysis and therefore we were unable to test the hypothesis that such an activator would express high, maltose-inducible levels of *LacZ*.

Transformants expressing the hybrid activator LexA-Mal63(1-283)p, in which residues 284-470 were deleted from the C-terminal end of Mal63p, constitutively expressed the *LacZ* reporter gene in glycerol/lactate grown cells to very high levels, comparable to the fully induced levels of expression seen with the deletions of residues 40-99 of Mal63p. This suggests that the transcription activation function lies within the N-terminal 283 residues of Mal63p, and that the deleted residues contain a negative regulatory domain which mediates the response to the inducer. Interestingly, in the presence of maltose, β-galactosidase activity levels were 3- to 4-fold lower than in the absence of maltose. Maltase gene expression also was reduced in maltose-grown cells but not to the same extent. The basis of this maltose-dependent, partial repression of the constitutive transcription activation is unknown but maltase expression in strains carrying constitutive MAL-activator mutations is also repressed in maltose-induced growth conditions as compared to uninduced conditions (Wojciechowicz, 1993; see below). This effect might result from an increase in intracellular levels of glucose due to maltose hydrolysis. LexA-Mal63(1-283)p also activated expression from the UAS_{MAL}. Cells carrying this

hybrid activator fermented maltose in the absence of the wildtype activator. Maltase expression levels were constitutive consistent with the results of the *LacZ* reporter expression. Since hybrid activators carrying deletions of residues 1-37, 40-60, 60-99 and 284-470 were all functional, this localizes the region required for transcription activation to residues 99-283.

To further localize the transcription activation domain, 67 amino acid residues were deleted from the C-terminal end of the constitutive hybrid activator to give LexA-Mal63p(1-216). This hybrid did not activate expression of the reporter gene. Additionally, residues 215-283 were not sufficient to activate transcription. The hybrid gene containing codons 215-283 of *MAL63* fused to *LexA*(1-87) was unable to activate *LacZ* expression, indicating that residues 215/216 are located within the activation domain.

Deletion of residues 250-300 resulted in a functional activator with reduced, but partially constitutive activity. Maltose-induced levels of *LacZ* expression were 3-fold lower than the full-length hybrid. There is a low level of β-galactosidase synthesis in glycerol/lactate medium and this is induced only 4-fold by maltose. This result, taken together with the results reported above further defines the region of Mal63p required for transcription activation to residues 99-250. Moreover, it suggests that residues 250-300 are involved in maltose regulation. Surprisingly, the strain carrying the

LexA/Mal63(Δ250-300)p hybrid was unable to ferment maltose and did not express maltase even under inducing conditions.

In an effort to localize the maltose-responsive negative regulatory domain within the C-terminal residues (284-470), residues 301-350, 351-401, 401-450 and 457-470 were independently deleted from the LexA-Mal63p hybrid activator. None of these deletion mutant hybrids were able to activate reporter gene expression under either condition. In addition strains carrying these hybrids were unable to ferment maltose. This result suggests that the deleted regions individually or together define a required functional domain(s) but contrasts with the *constitutive* activity of the LexA-Mal63(1-283)p hybrid. We interpret this apparently conflicting result to indicate two possibilities which are not mutually exclusive; i) 50 residue deletions in the C-terminal region destroy the folding pattern of the region and normal function(s) in this region is lost.

ii) The C-terminal region (residues 283-470) are not required for the *trans*-activation function of Mal63p but this region serves a dual regulatory role. First, in the absence of maltose, the C-terminal region represses *trans*-activation but, second, in the presence of maltose it is required to undo the repressing effects of this region. This positive function is not required in this LexA-fusion construction when residues 283-470 (and their repressing function) are entirely deleted.

The 14 C-terminal residues of Mal63p are highly acidic (6

out of 14 residues). Acidic residues have been associated with transcription activation in several activators (Mitchell and Ptashne 1989). Since deletion of 457-470 resulted in a transcriptionally inactive hybrid activator, the possibility was raised that this C-terminal acidic region might function as a second activation domain. To test this, we constructed a series of hybrid activators containing deletions of the previously defined transactivation domain in residues 100-250. Neither LexA-Mal63(Δ100-149)p, LexA-Mal63(150-470)p nor LexA-Mal63(215-470)p, all of which contain this acidic C-terminal region, could activate transcription of the *LacZ* reporter gene. *LexA-MAL63(395-470)* was also constructed but a stable fusion product could not be detected in Western analysis.

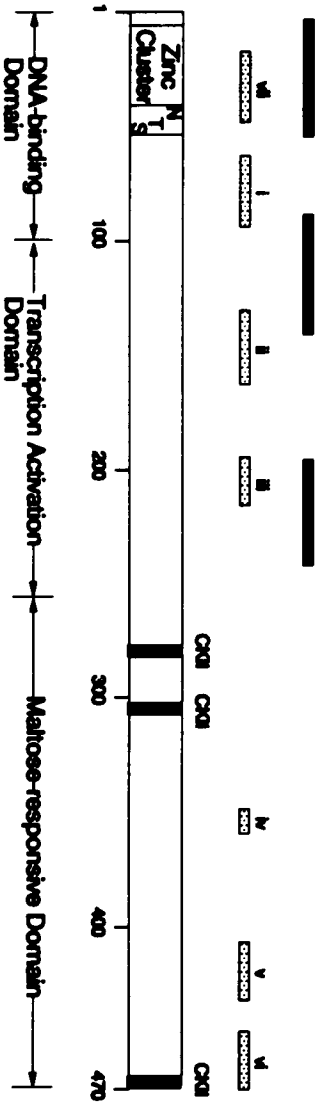
These results suggest that this C-terminal acidic region is transcriptionally inactive. However, in view of the fact that residues 284-470 contain a maltose-responsive negative regulatory region, it is possible that this domain is acting in these constructions to inhibit an otherwise functional transcription activation domain located in the 14 terminal residues. To test this possibility, we constructed a fusion gene expressing the hybrid activator, LexA-Mal43-C(215-470)p, which contains residues 215-470 of the dominant, constitutive *MAL43-C* activator gene. Residues 215-470 of Mal43-Cp are 90% identical to Mal63p and contain the alterations responsible for the dominant, constitutive phenotype of this activator implying that any negative regulatory function of this region

is inactive (see below). The LexA-Mal43-C(215-470) protein was unable to activate *LacZ* expression. Taken together these results strongly suggest that the C-terminal 14 residues of Mal63p do not represent a second transcription activation domain but are required for some other critical function.

In summary, we have extended the region of Mal63p required for binding to the UAS_{MAL} to include residues 40-99 which are adjacent to the zinc cluster (residues 8-38)(Figure 1). We have localized the transcription activation function of Mal63p to a single region from residues 99-250. We also have identified a maltose-responsive, negative regulatory region within residues 284-470 and have shown that residues 250-300 appear to be involved in this function. Finally, while residues 300-470 contain this inhibitory domain they also contain function(s) required for induction, that is, release from the inactive state. A summary of the functional domains of Mal63p is shown in Figure 1.

Figure 1. Summary of the functional domains of the Mal63p activator.

Numbers below the diagram represent amino acid residues. The DNA-binding domain containing the cysteine-rich zinc cluster, and the transcription activation and maltose-responsive domains are indicated. A putative nuclear targeting signal (NTS) is also indicated. Casein kinase II phosphorylation sites (CKII) are indicated in the diagram by black boxes. Topoisomerase II homology regions are indicated by the stippled boxes immediately above the figure. Roman numerals above the stippled boxes indicate the order in which these sequences appear in topoisomerase II. CASUC1p homology regions are indicated by the black boxes at the top of the diagram.


DNA-binding Domain
Transcription Activation Domain
Maltose-responsive Domain
1
100
200
300
400
470
Zinc Cluster
N
T
S
CXII
CXII
CXII
VII
I
II
III
IV
V
VI

B. Molecular genetic analysis of a constitutive MAL-activator mutation.

To further characterize the maltose-responsive domain in the *MAL*-activator protein we decided to characterize an already existing, constitutive allele of *MAL43* isolated from the yeast strain 1403-7A. Strain 1403-7A had been described previously by Khan and Eaton (1971) as a maltose-fermenting yeast strain whose synthesis of maltase was constitutive and partially insensitive to glucose-repression. Results of genetic analysis of 1403-7A established that the constitutive, glucose-repression-insensitive phenotype of strain 1403-7A was dominant and linked to the *MAL4* locus. The *MAL43* gene from 1403-7A was cloned and shown to encode a constitutive MAL-activator alteration referred to as *MAL43-C* (Charron and Michels, 1987). Additionally, the cloned *MAL43-C* allele was shown to be dominant to the inducible *MAL63*. To determine the molecular genetic basis of its mutant phenotype we decided to sequence the *MAL43*-C gene, and to compare the predicted amino acid sequence to that of the wildtype, inducible activator. In this way any altered residue(s) could be readily identified thereby localizing the maltose-responsive domain. However, since no inducible *MAL4* strains were available in the strain collections, we were forced to compare the Mal43-Cp sequence to that of the wildtype, inducible Mal63 protein (Kim and Michels, 1988). In addition, based on restriction map comparison of the *MAL* loci, the *MAL4* locus appears to be more

closely related, evolutionarily, to the *MAL2* locus (Charron *et al.*, 1989). For this reason we also compared the Mal43-Cp sequence to the predicted amino acid sequence of the wildtype, inducible Mal23p.

The nucleotide sequence of *MAL43*-C and *MAL23* are shown in Figure 2 and Figure 3, respectively. Both genes contain a single, 1.41 kbp open reading frame encoding a 470 amino acid residue protein, and share 95% amino acid sequence identity. The nucleotide sequence of 170 basepairs of upstream sequence immediately 5′ to the ATG start codon of *MAL23* and *MAL43-C* are 99% identical. A comparison of the predicted amino acid sequences of Mal63p, Mal23p and Mal43-Cp is shown in Figure 4. Table 4 lists the amino acid residues that differ in the three activator proteins. There are 22 residue differences between Mal23p and Mal43-Cp and eighteen of these occur between residues 215 to 470. Of the 31 amino acid residue differences between Mal63p and Mal43-Cp, 27 lie between positions 215 and 470 in the C-terminal region of the protein, and, of these, 14 are identical to residues found in the inducible Mal23p. Since these 14 residues are the same in both the wildtype, inducible activator (Mal23p) and the constitutive activator (Mal43-Cp) this suggests that these residues alone may not be important to the mutant phenotype of Mal43-Cp. However, one or more of these residues may be important in the "context" of several of the other Mal43-Cp alterations and together these may result in the constitutive phenotype. The other C-

Figure 2. Nucleotide sequence of *MAL43* and the deduced amino acid sequence of the protein (Mal43-Cp).

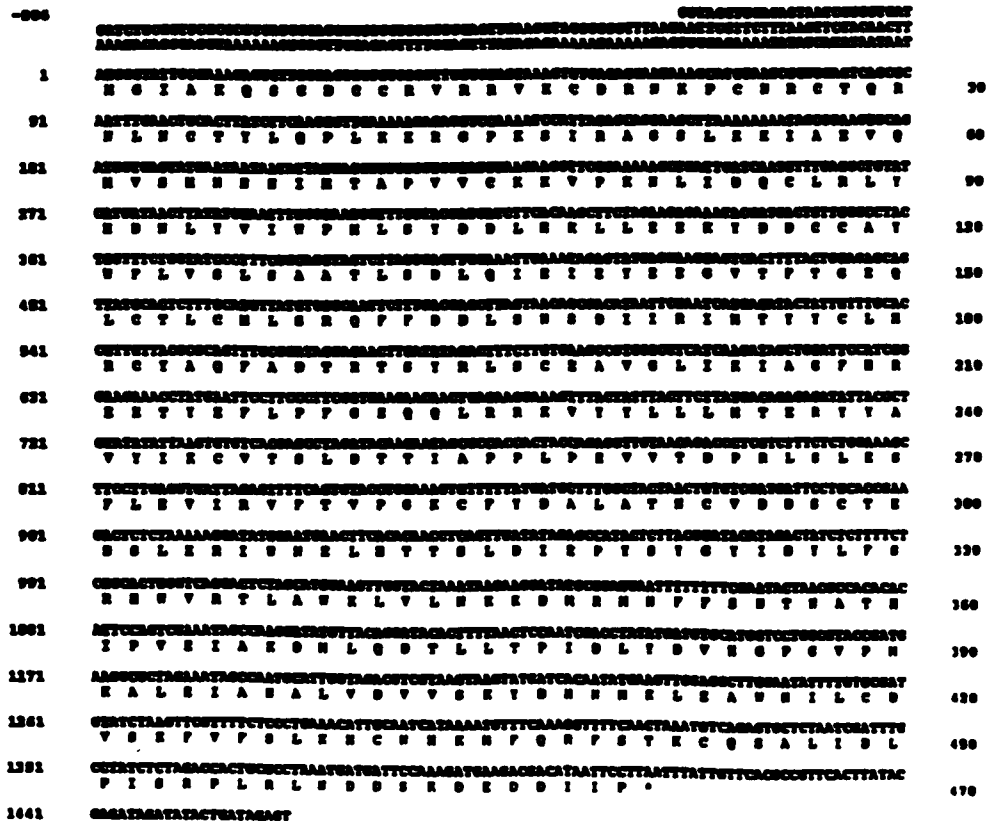
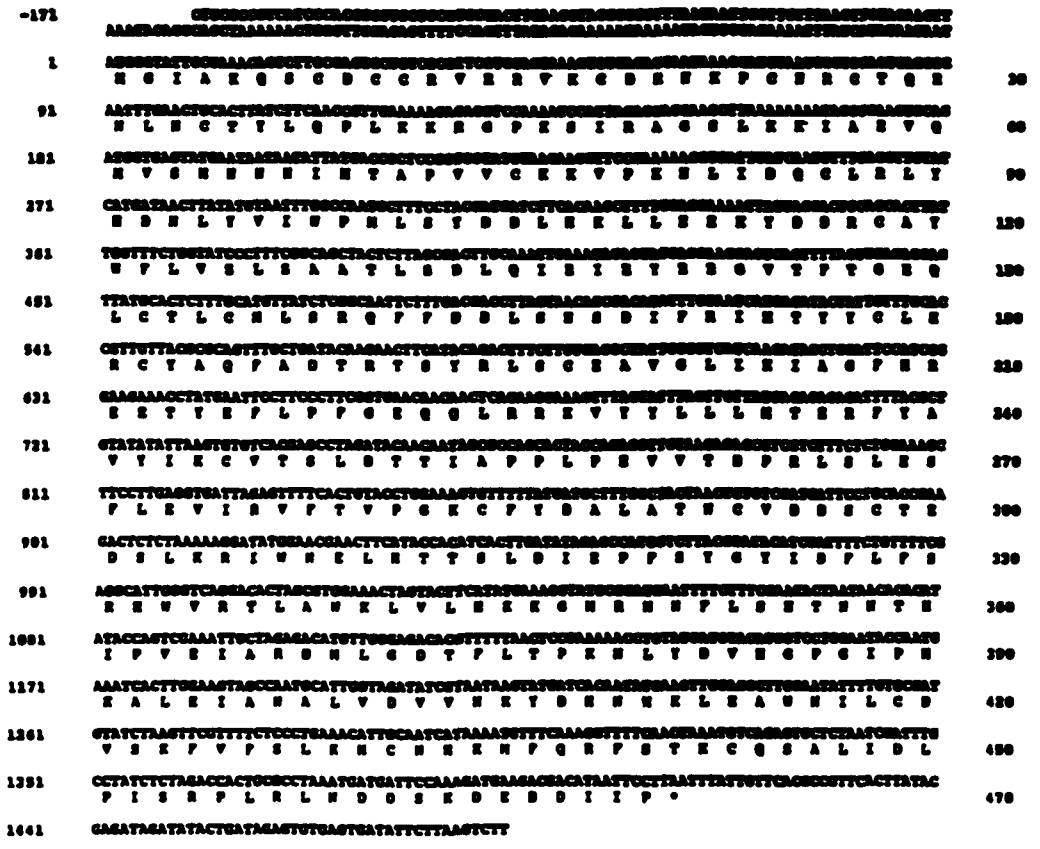


Figure 3. Nucleotide sequence of *MAL23* and deduced amino acid sequence of the Mal23p protein.



terminal alterations are not clustered together but are scattered over the C-terminal region. While some of the alterations represent conservative changes others are non-conservative and result in significant changes in size and/or charge in the residue at that position (see Table 4). Since gross amino acid changes in the functional domain of a protein can result in an alteration in the domain function (Branden and Tooze, 1991) it is likely that one or more of the amino acid changes occurring in Mal43-Cp may have resulted in the gain of function mutation giving *MAL43-C* its dominant mutant phenotype.

To localize which one (or ones) of the altered residues in Mal43-Cp is responsible for its constitutive phenotype we decided to create hybrid molecules in which one or a few of the inducible Mal63p residues are converted to the corresponding Mal43-Cp residues. Table 5 shows the structure of each hybrid gene. Above the table is a cartoon representing the MAL-activator. Amino acid differences between Mal63p and Mal43-Cp are indicated by a vertical line. As is shown in Table 4, the variable residues are clustered in the C-terminal region. The structure of each hybrid is indicated as follows. In *MAL63/43(341-361)*, codons 341-361 of *MAL63* were changed so that the product of this hybrid gene will have the same amino acid sequence as Mal43-Cp in the region from residues 341-361, and, by referring to Table 4, one can see that the following changes were made: H344N,

Figure 4. Comparison of the deduced amino acid sequences of Mal63p, Mal43-Cp and Mal23p.

The deduced sequence of Mal63p was previously reported (Kim and Michels, 1988). The residues which differ between the three proteins are indicated.

```
MAL63    M G I A K Q S C D C C R V R R V K C D R N K P C N R C I Q R N L N C T Y L Q P L K K R G P K S I R A G S L K K I A E V Q   60
MAL43-C  M G I A K Q S C D C C R V R R V K C D R N K P C N R C T Q R N L N C T Y L Q P L K K R G P K S I R A G S L K K I A E V Q   60
MAL23    M G I A K Q S C D C C R V R R V K C D R N K P C N R C T Q R N L N C T Y L Q P L K K R G P K S I R A G S L K K I A E V Q   60

MAL63    N V S N N N N N I N A A P V V C K K V P K N L I D Q C L R L Y H D N L Y V I W P N L S Y D D L N K L L E E K Y D D R C A Y  120
MAL43-C  N V S N N N N N I N T A P V V C K K V P K N L I D Q C L R L Y H D N L Y V I W P N L S Y D D L N K L L E E K Y D D C C A Y  120
MAL23    N V S N N N N N I N T A P V V C K K V P K N L I D Q C L R L Y H D N L Y V I W P N L S Y D D L N K L L E E N Y E D C S T Y  120

MAL63    W F L V S L S A A T L S D L Q I E I E Y E E G V T F T G E Q L C T L C N L S R Q F F D D L S N S D I F R I N T Y Y C L N  180
MAL43-C  W F L V S L S A A T L S D L Q I E I E Y E E G V T F T G E Q L C T L C N L S R Q F F D D L S N S D I I R I N T Y Y C L N  180
MAL23    W F L V S L S A A T L S D L Q I E I E Y E E G V T F T G E Q L C T L C N L S R Q F F D D L S N S D I F R I N T Y Y C L N  180

MAL63    R C Y A Q F A D T R T S Y R L S C E A V G L I K I A G F H R E E T Y E F L P F G E Q Q L R R K V Y Y L L L N T Q R F Y A  240
MAL43-C  R C Y A Q F A D T R T S Y R L S C E A V G L I K I A G F H R E E T Y E F L P F G E Q Q L R R K V Y Y L L L N T Q R Y Y A  240
MAL23    R C Y A Q F A D T R T S Y R L S C E A V G L I K I A G F H R E E T Y E F L P F G E Q Q L R R K V Y Y L L L N T Q R F Y A  240

MAL63    V Y I K C V T S L D A T I A P P L P E V V T D P R L S L E S F L E V I R V F T I P G K C F Y D A L A T N C V D D S C T E  300
MAL43-C  V Y I K C V T S L D T T I A P P L P E V V T D P R L S L E S F L E V I R V F T V P G K C F Y D A L A T N C V D D S C T E  300
MAL23    V Y I K C V T S L D T T I A P P L P E V V T D P R L S L E S F L E V I R V F T V P G K C F Y D A L A T N C V D D S C T E  300

MAL63    D S L K R I R N D L N T T S L D I E P W S Y G Y I D F L F S R N W V R T L A W K L V L N N K G N R N N F L S N T N N T N  360
MAL43-C  D S L K R I W N D L N T T S L D I E P Y S Y G Y I D Y L F S R N W V R T L A W K L V L N K K D N R N N F F S N T N A T N  360
MAL23    D S L K R I W N D L N T T S L D I E P W S Y G Y I D F L F S R N W V R T L A W K L V L N N K G N R N N F L S N T N N T N  360

MAL63    I P V E I A R D N L G D T F L T P K N L Y D V N G P G I P N K A L E I A N A L V D V V N K Y D N N N K L E A W N V L Y D  420
MAL43-C  I P V E I A K D N L Q D T L L T P I D L Y D V N G P G V P N K A L E I A N A L V D V V S K Y D N N N K L E A W N I L C D  420
MAL23    I P V E I A R D N L G D T F L T P K N L Y D V N G P G I P N K S L E V A N A L V D I V N K Y D N N N K L E A W N I L C D  420

MAL63    V S K F V F S L K N C N N K N F D R F S T K C Q G A L I T L P I S K P L Q L N D N S K D Q D D I I P                      470
MAL43-C  V S K F V F S L K N C N N K N F Q R F S T K C Q S A L I D L P I S R P L R L N D D S K D Q D D I I P                      470
MAL23    V S K F V F S L K N C N N K N F Q R F S T K C Q S A L I D L P I S R P L R L N D D S K D Q D D I I P                      470
```

Table 4. Comparison of the variant amino acid residues that occur between the Mal63p, Mal23p and Mal43-Cp activator proteins.

Numbers represent codon positions within the 470 residue open reading frame. The single letter codes are used to indicate the amino acids.

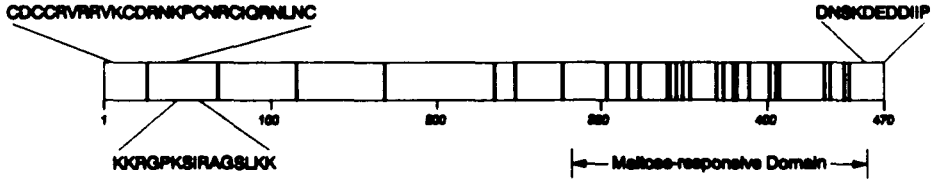
	VARIANT AMINO ACID RESIDUES		
Position	Mal63p	Mal23p	Mal43-Cp
28	I	T	T
70	A	T	T
113	K	N	K
115	D	E	D
117	R	C	C
118	S	C	C
119	A	T	A
171	F	F	I
238	F	F	Y
251	A	T	T
280	I	V	V
307	R	W	W
320	W	W	Y
327	F	F	Y
344	H	H	N
345	M	M	K
347	G	G	D
353	L	L	F
358	N	N	A
367	R	R	K
371	G	G	Q
374	F	F	L
378	K	K	I
379	N	N	D
388	I	I	V
392	A	S	A
395	I	V	I
402	V	I	V
404	N	N	S
417	V	I	I
419	Y	C	C
433	N	H	H
437	D	Q	Q
445	G	S	S
449	T	D	D
454	K	R	R
457	Q	R	R
461	N	D	D

M345K, G347D, L353F, N358A. Each MAL63/MAL43-C hybrid gene was cloned into the CEN vector YCp50, transformed into strain 340-2A which lacks a MAL-activator gene and maltase expression was assayed in transformants grown under uninduced, induced and repressed conditions. The results are shown in Table 5. There was virtually no distinction between the levels of maltase expressed in cells carrying either the *MAL43*-C gene or the hybrid gene *MAL63(1-215)/43-C(216-470)*. Cells carrying this construction, which encodes a hybrid protein containing 27 of the 31 variant residues found in Mal43-Cp, showed high levels of maltase gene expression in the absence of maltose and were also partially glucose-repression-insensitive. Thus, the Mal43-Cp alterations responsible for the constitutive phenotype are in the C-terminal half of the protein.

Efforts to localize the essential alteration(s) to a more defined region failed. None of the hybrid genes carrying fewer of the C-terminal alterations were constitutive. Alternately, the *MAL43-C* hybrids in which residues Y320W, Y327F and S404N were converted to the corresponding Mal63p residues retained their constitutive phenotype and expression levels were similar to those obtained with the *MAL43-C* allele, again suggesting that these residues by themselves are not sufficient to alter the phenotype of the molecule. In summary, only the alteration of all of the 27 residues of Mal63p to the sequence found in Mal43-Cp is sufficient to produce a constitutive activator.

Table 5. Activation of maltase expression by *MAL63/MAL43-C* hybrid genes.

Plasmids containing *MAL63/MAL43-C* hybrid genes were constructed in YCp50 as described in the Materials and Methods and transformed into strain 340-2A which lacks a functional activator gene. Conditions for maltase assays were the same as presented in Table 3, except that cells were grown in SM media lacking uracil. Glucose medium consisted of SM + 3% glycerol + 2% lactate + 2% glucose. The cartoon drawing represents the MAL-activator with the N-terminal zinc cluster, the proposed nuclear targeting sequence and C-terminal acidic regions indicated. The vertical lines represent the positions of amino acid residues that differ between Mal63p and Mal43-Cp.



	Maltase Activity		
	Gly/Lac	Maltose	Glucose
YCp50	18	36	8
YCpMAL63	26	768	7
YCpMAL43-C	971	871	277
YCp63(1-215)/43(216-470)	890	813	297
YCpMAL63/43(341-361)	29	802	8
YCpMAL63/43(361-385)	65	909	15
YCpMAL63/43(439-466)	78	714	13
YCpMAL63/43(361-385, 439-466)	77	1395	2
YCpMAL43(Y320W, Y327F)	672	674	258
YCpMAL43(Y320W, Y327F, S404N)	947	738	375

C. Over-expression of *MAL63*.

Several transcription activators in *Saccharomyces* are regulated by inhibitory factors. In some cases, these factors have been shown to physically interact with the activator, and to inhibit transcription of the target genes under uninduced or repressed conditions. For example, the Gal4p transcription activator, which stimulates the expression of the galactose fermentation genes in the presence of galactose, physically interacts with and is inhibited by Gal80p in the absence of galactose. In the presence of galactose, this inhibitory effect is relieved. Since Gal80p is present in limiting quantities, its negative effects can be overcome by increasing the concentration of the activator molecules. Johnston and Hopper (1984) showed that overexpression of *GAL4* using a high copy plasmid vector resulted in constitutive, partially glucose-repression-insensitive expression of the *GAL* structural genes. Other activators however are regulated by mechanisms that do not involve a titratable inhibitor protein. For example, overexpression of the Put3 activator, required for expression of the proline utilization enzymes, does not result in constitutive expression of the target genes.

To determine whether Mal63p is regulated by a titratable inhibitory factor, we overexpressed *MAL63* by increasing its copy number in the high copy number plasmid vector, YEp352. In yeast cells, this vector has been reported to be present in excess of 50 copies as opposed to 1 to 2 copies of YCp50 per

cell. Using similar vectors, Johnston and Hopper (1984) were able to titrate the limiting quantities of Gal80p and demonstrate constitutive *GAL* gene expression. *MAL63* was cloned into YCp50, and YEp352. Both constructs and the corresponding control plasmids without inserts were transformed into yeast strain 340-2A, which contains the structural genes required for maltose fermentation at the *MAL1* locus but lacks any copies of the MAL-activator gene, and maltase expression determined following growth under uninduced, induced and glucose-repressed conditions. The results are shown in Table 6.

Under uninduced conditions there is no significant difference in the maltase activity of cells bearing either single copy or multiple copies of *MAL63*. Under induced conditions, only a 1.5 to 2.0 fold increase in the maltase expression is seen in cells overexpressing the activator. The maltase activity of cells carrying the *MAL43-C* constitutive activator is included here for comparison. These results suggest that no titratable inhibitor exists in this system.

Table 6. Effect of increased copy number of *MAL63* on maltase gene expression in strain 340-2A.

Plasmids were constructed as described in Materials and Methods and were transformed into yeast host strain 340-2A, genotype *AGT1 MAL12 Δmal13* (Charron *et al.*, 1988).

	Maltase Activity (nmoles PNPG/min/mg protein)		
Plasmid	Gly/Lac	Maltose	Glucose
YCp50	26	67	5
YCpMAL63	18	831	5
YEpMAL63	31	1348	11
YCpMAL43-C	971	871	281

D. Two-hybrid analysis of Mal63p/Mal63p interaction.

The results reported above clearly indicate that the C-terminal residues 283-470 mediate a negative regulatory function which represses the ability of Mal63p to activate *MAL* structural gene expression in the absence of the inducer, maltose. Overexpression of Mal63p failed to uncover any titratable Gal80p-like inhibitory factors that might interact with this region of Mal63p. One possible alternate explanation for the negative regulation of Mal63p activity is that, in the presence of maltose, Mal63p undergoes a conformational change from an inactive to an active form which now allows the protein to stimulate gene expression. This suggests the possibility that different parts of the protein may interact under different physiological conditions. To test this model we used the Two-Hybrid System of Fields and Song (1989). In this system two hybrid proteins are synthesized *in vivo* in yeast. One hybrid contains a fusion between protein-X and the LexAp DNA-binding domain (residues 1-87), while the other contains a fusion between protein-Y and the Gal4p transcription activation domain (residues 768-881). Interaction between proteins X and Y results in reconstitution of a complete transcription activator that can bind to a LexA operator site and activate transcription of a downstream (*LacZ*) reporter gene.

To utilize the Two-hybrid System, both hybrid constructions must be inactive for reporter gene expression

when present alone. Thus, it was necessary to use non-functional deletion mutations of the *LexA-MAL63* fusions (see above). Two hybrid proteins containing either residues 1-300 or 199-470 of Mal63p fused, in frame, to the Gal4p(AD) were constructed. The *LexA(BD)-MAL63* and *GAL4(AD)-MAL63* hybrid genes were transformed, in all possible combinations, into strain YPH500 which carries a *LacZ* reporter with 6 LexA-binding sites in the promoter. *LacZ* expression was monitored by a plate assay on the appropriate minimal media plates containing X-Gal and either maltose, glucose or glycerol/lactate as carbon sources. Reporter gene expression, indicating an interaction between the Mal63 protein fragments, could be detected by the formation of blue dye (hydrolysed X-Gal). The results are shown in Table 7. None of the combinations of hybrids were able to activate expression of *LacZ*. Plasmids carrying the *LexA-SNF4* and *GAD-SNF1* fusions genes which encode interacting hybrid proteins were used as a positive control.

Table 7. Effect of *LexA-MAL63* and *GAL4(AD)-MAL63* Two-Hybrid fusions on β-galactosidase expression in YPH500.

Hybrid genes were constructed using the *LexA(1-87)* DNA-binding domain and the *GAL4(768-881)* transcription activation domain (referred to as GAD) and the plasmids transformed into YPH500 as described in Materials and Methods. *LacZ* reporter gene expression was monitored by the plate test assay (described in Bohen and Yamamoto, 1993) on SM media lacking uracil, histidine and leucine and containing the indicated carbon sources.

Plasmid 1	Plasmid 2	Gly/lac	Maltose
LexA	GAD	White	White
LexA-MAL63(2-456)	GAD-MAL63(1-300)	White	White
LexA-MAL63(2-456)	GAD-MAL63(199-470)	White	White
LexA-MAL63(1-215)	GAD-MAL63(1-300)	White	White
LexA-MAL63(1-215)	GAD-MAL63(199-470)	White	White
LexA-MAL63(215-470)	GAD-MAL63(1-300)	White	White
LexA-MAL63(215-470)	GAD-MAL63(199-470)	White	White
LexA	GAD-MAL63(1-300)	White	White
LexA	GAD-MAL63(199-470)	White	White
LexA-MAL63(2-456)	GAD	White	White
LexA-MAL63(1-215)	GAD	White	White
LexA-MAL63(215-470)	GAD	White	White
LexA-SNF4	GAD-SNF1	Blue	Blue

Discussion

Many transcription activators that have been studied appear to consist of several separable domains responsible for nuclear localization, DNA-binding, inducer response and transcription activation. Previous studies identified an N-terminally located DNA-binding zinc-cluster in residues 8-38 of Mal63p (Kim and Michels, 1988; Kim, 1992), but other functional domains remained unidentified. Our goal here is to localize the other functional domains in the *Saccharomyces* MAL-activator. For this, we assayed the ability of a series of LexA-Mal63p hybrids containing Mal63p deletion mutations, to activate expression of a $LexA_{op}$-*LacZ* reporter. Additionally, we carried out a molecular genetic analysis of a dominant, constitutive MAL-activator mutation, *MAL43-C*. Our results indicate that, like other yeast *trans*-activators, the region C-terminal to the zinc cluster is required for DNA-binding (residues 40-99); the transcription activation domain is located in residues 99-250; the C-terminal residues 250-470 contain the maltose-responsive regulatory domain which both represses in the absence of inducer and, in the presence of inducer, is required for function/induction. Unlike other *trans*-activators, the transcription activation region, although containing acidic residues often associated with activation domains, appears to be more complex. In addition,

unlike many other *trans*-activators, the regulatory domain could not be localized to a short, discrete and separable sequence.

The DNA-binding domain of Mal63p

As expected, the LexA/Mal63p hybrid protein from which the Mal63p, C_6 zinc cluster had been deleted (residues 1-38) was still functional as an inducible activator of reporter gene expression but not *MAL* gene expression. This is consistent with previous results which indicated that this region is required for DNA-binding (Kim,1992). Our results also suggest that, unlike certain other activators, these residues are not involved in the transcription activation function. Recent data suggests that, in some yeast activators, residues in the zinc cluster region of the DNA-binding domain are involved in the activation function. This was seen in the Hap1p activator protein where mutations in Ser60, located immediately N-terminal to the first cysteine residue (61) of the zinc cluster region, resulted in a constitutive phenotype (Kim and Guarente, 1989), or altered the expression of different target genes (Fytlovich *et al.*, 1993). In the Adr1p activator protein seven different zinc-finger mutations, which resulted in the inability of the protein to bind to DNA, also resulted in reduced transcription activation function when the mutant activators were fused to the LexA DNA-binding domain (Cook, *et al.*, 1994). The precise role of these residues in the *trans*-

activation function is still unknown, however, it is possible that the region plays a structural role in promoting or inhibiting efficient interaction between the activator and other promoter-bound general transcription factors to alter the expression of different target genes under different physiological conditions (Fytlovich *et al.*, 1993).

The region encompassing residues 40-99 of Mal63p appears not to be essential for transcription activation or induction as deletions of residues 40-66 and 60-99 resulted in activators capable of maltose-induced expression of *LacZ*. In both cases *LacZ* expression was 8- to 10-fold above wild type levels. This increased expression could imply that there is an inhibitory role mediated by this region with respect to *LacZ* reporter expression. Perhaps, this region antagonizes proper binding of the hybrid to the LexA operator. Alternatively, it is possible that this region folds improperly in the hybrid activator and results in reduced contact with the general transcription factors. Studies by Golemis and Brent (1992) suggest that proteins fused to LexA generally antagonize operator binding by the LexA moiety. Relative to $LexA_{1-87}$ alone, nearly all LexA hybrids tested bind poorly to the operator, even when present in excess, and fail to form *in vitro* complexes with the $LexA_{op}$ (Golemis and Barent, 1992). Thus, deletion of residues 40-99 from Mal63p may allow LexA-Mal63p hybrids to bind optimally to the $LexA_{op}$.

Our finding that strains carrying the Δ40-66 and Δ60-99

hybrid activators which exhibit elevated *LacZ* expression are unable to ferment maltose suggests that the deleted regions, which are adjacent to the zinc cluster, are required for sequence specific binding to the UAS_{MAL}. Whether this region is directly involved with the DNA-binding or contains a dimerization domain required for UAS_{MAL} binding has not been determined. Regions of sequence-specific transcription activators which confer binding specificity have been shown to be adjacent to the DNA-binding domain (Corton and Johnston, 1989; Mitchell and Tjian, 1989; Reece and Ptashne, 1993). In studies performed on yeast transcriptional activators, Gal4p, Put3p and Ppr1p, which contain C_6 zinc clusters similar to that in Mal63p, residues in the region immediately adjacent to the C_6 zinc cluster were shown to confer sequence specificity on the DNA-binding of the zinc cluster. This specificity region includes a short 8-10 residue linker region (immediately C-terminal to cysteine 6), and the adjacent 40-60 residues which form part of the dimerization domain. Thus, chimeric activators containing the Gal4p zinc cluster (residues 1-38), or the Ppr1p zinc cluster (residues 29-61) fused to the linker-dimerization region of Put3p (residues 61-126) both recognize and bind strongly to a Put3p binding site, but do not bind to Gal4p or Ppr1p binding sites (Reece and Ptashne, 1993). In addition, the dimerization regions in these proteins are thought to form dimers through interaction of coiled-coil structures, the formation of which are based on

the hydrophobic heptad repeat or leucine zipper. Examination of the sequence of residues 40-99 of Mal63p shows that no sequence or structural homology exists between its putative linker-dimerization region and those of Gal4p, Put3p and Ppr1p. This region of Mal63p is incapable of forming the usual leucine zipper which contains a leucine residue or other hydrophobic residue at every seventh position. However, this does not diminish the possibility that this region in Mal63p is involved in dimerization, since protein-protein interactions, in many other cases, occur in the absence of leucine zipper-like interactions. Regardless of its precise structure, our results indicate that this region is involved in binding to the UAS_{MAL}. How this function is carried out and why this region appears to inhibit *LacZ* reporter expression must be explored further.

The transcription activation domain of Mal63p

Our results indicate that the transcription activation domain of Mal63p lies between residues 99-250. Studies of other transcription activators have revealed the existence of several general types of transcription activation domains including; proline-rich regions (for example, Sp1), glutamine-rich regions (for example, CTF/NF-1), acidic regions capable of forming α-helices (for example, Gcn4p); and β-sheet structures (for example, Gal4p) (Ma and Ptashne, 1987; Mitchell and Tjian, 1989; Van Hoy *et al.*, 1993). Examination

of the sequence of Mal63p between residues 99-250 reveals two acidic regions, residues 104-116 and 133-149, that might qualify as transcription activation domains, however, our results suggest that the activation domain of Mal63p is more complex since the hybrid protein LexA-Mal63p(1-215) which contains both these acidic regions was unable to activate *LacZ* expression (Kim and Michels, 1989; Mitchell and Tjian, 1989).

The importance of the acidic and α-helical nature of some activation domains have been called into question recently by studies of Gal4p activation domain. Data from these studies where altered acidic residues were still functional, suggest that the acidic nature of the activation domain is unimportant for function (Leuther *et al.*, 1993). In addition, evidence from circular dichroism spectroscopy suggests that the domain forms a β-sheet rather than an α-helix (Van Hoy *et al.*, 1993). Studies with other activators also give similar results. A mutant p53 protein in which each of the acidic residues in the activation domain had been altered was still transcriptionally active, suggesting that the transcription activation function is due to some other feature of the domain (reviewed in Prives, 1994).

Residues 99-250 of Mal63p may contain a previously uncharacterized, unusual type of activation domain. Alternatively, this region could provide surfaces which interact with transcription co-activators. Co-activators or adaptors are recently identified transcription factors which

do not bind to DNA, but which interact with sequence-specific, DNA-binding transcription activators and mediate an interaction with the transcripiton machinery (Berger *et al.*, 1992; Piña *et al.*, 1993; Ge and Roeder, 1994; Kretzschmar *et al.*, 1994; Li *et al.*, 1994). In all cases these factors have been shown to physically interact with transcription activators, or with components of the general transcription machinery (for example, TFIID).

The functional importance of this proposed transcription activation region is further highlighted by the finding that several uninducible *mal63* point mutations map to this region (ten Berge *et al.*, 1973; J. Kim, unpublished results). One mutant allele, 10U, contains a Gly148 to Glu change while the other, SW25, contains two alterations, Gln85 to Pro, and Ala119 to Val. It has not been determined which of the two altered residues in SW25 is responsible for the noninducible phenotype but a temperature sensitive revertant, SW25R, contained a Cys86 to Tyr alteration, suggesting that the conservative Ala119 to Val change may not be the critical one. Finally, mutation SW14 is an Ala254 to Thr alteration.

The transcription activation domain and zinc cluster region of Mal63p also exhibit homology to a recently reported *Candida albicans* gene, *CASUC1* (Kelly and Kwon-Chung, 1992). *CASUC1*, isolated in a search for sucrose utilization genes, encodes a putative transcription factor with the ability to complement *MAL*-activator gene mutations in *Saccharomyces*.

Maltase expression is induced 5-fold in transformants carrying *CASUC1* as compared to 15-fold induction seen in transformants expressing *MAL63*. The zinc cluster regions of both proteins show a great deal of sequence homology (51% identity) extending past the cysteine-rich region (residues 8-34) to residue 46 and thus including what Reece and Ptashne (1994) refer to as the linker. No significant sequence homology is seen between these proteins in residues 46-110, which we have shown are essential for binding of Mal63p to the UAS_{MAL} and which Reece and Ptashne (1994) demonstrated contains the specificity/dimerization domain in Gal4p, Ppr1p and Put3p. Perusal of this region in Mal63p and CASUC1p reveals about 35% hydrophobic residues (leucine, isoleucine, valine and alanine) in both proteins. Perhaps this hydrophobic sequence is structurally homologous in the two proteins and this, as yet undefined, structure provides the specificity/dimerization function required for the DNA binding-site selection.

Residues 96-142 of CASUC1p are 38% identical and 59% similar to Mal63p residues 95-145, and CASUC1p residues 200-241 are 44% identical and 71% similar to Mal63p residues 198-239. Thus, the region of Mal63p, which we have shown is required for transcription activation, is conserved in CASUC1p. It is likely that this region of both proteins has a similar function. The lack of homology in the C-terminal region of these proteins which we have shown is responsible for maltose-regulation in Mal63p, is interesting in view of

the fact that both proteins respond to maltose induction. At this point, too little is known about the mechanism of maltose-induction and the signal generated by maltose to comment further on this finding.

Finally, in concluding that the Mal63p transcription activation domain lies between residues 99-250 we must keep one caveat in mind. It is possible that this region also contains a dimerization domain. Data suggest that $LexA_{1-87}$ can bind to isolated operator half-sites as a monomer (Ptashne, 1978; Keegan, *et al.*, 1986) and that $LexA_{1-87}$-activator chimeras may not need dimerization domains to activate reporter gene expression from the LexA binding-site (Kim and Little, 1992). Other studies suggest that some heterologous dimerization domains (for example, Gal4p and Gcn4p) cannot substitute for the $LexA_{88-202}$ dimerization domain in correctly positioning LexA on the $LexA_{op}$ for optimal binding and efficient reporter gene expression (Golemis and Brent, 1992). Thus, while we feel confident that residues 99-250 contain a transcription activation domain, we cannot rule out the possibility that this region also serves to form an active dimerization structure.

<u>The regulatory domain of Mal63p.</u>

Several lines of evidence indicate that residues 300-470 are involved in the negative regulation of Mal63p function in the absence of maltose. First, the mutational alterations

responsible for the constitutive phenotype of *MAL43-C* are dispersed in the region encoding residues 238-460. Second, the truncation construct of *LexA-MAL63* with a termination codon at 284 produced a constitutive activator of both *MAL* gene and *LacZ* expression. Third, nonsense mutations at codons 283 (*MAL64-R10*) and 307 (*MAL64-C2*) of *mal64*, a *MAL6*-linked nonfunctional homologue of *MAL63* (85% identical) have a dominant constitutive phenotype (Wojciechowicz, 1992). Finally, a MAL64/63p hybrid activator in which residues 1-215 of mal64p are fused to residues 216-470 of Mal63p is inducible.

We were unable to localize this negative regulatory function to a discrete domain within the protein as has been done for several other *Saccharomyces* transcription activators including Adr1p, Ste12p and Leu3p. For these activators deletion and/or mutation of defined regions results in constitutive activators and allowed the identification of an inducer binding site (Hap1; Pfeiffer *et al.*, 1989), a phosphorylation site shown to be the target of a regulatory signal transduction pathway (Ste12p; Kirkman-Correia, 1993), and a region of intramolecular protein-protein interaction resulting in the regulation of transcription activation (Leu3p; Zhou *et al.*, 1990). No discrete regulatory sites were identified by our studies of the LexA-Mal63p fusions and the Two-Hybrid analysis did not support the possibility of Mal63p/Mal63p protein interaction.

In other regulators, a second component is used as a negative regulator. Constitutive mutations in Gal4p have identified a regulatory domain within the C-terminal 30 residues which interacts with Gal80p, a negative regulator of Gal4p transcription activation. This regulatory domain overlaps with the C-terminal transcription activation domain of Gal4p. Deletion of *GAL80* or overexpression of *GAL4* also result in constitutive Gal4p activity (Johnston & Carlson, 1993). Therefore, induction of the galactose fermentative genes in the presence of galactose involves relieving the inhibitory effects of Gal80p on the Gal4p activator. Another example of a similarly regulated system is the repressible acid phosphatase system. Pho4p activator protein binds upstream of the three rAPase genes and stimulates their expression when intracellular phosphate levels are low. However, when phosphate levels are high, Pho4p activity is inhibited by interaction with Pho80p. Overexpression of *PHO4*, or deletion of *PHO80* results in constitutive expression of the rAPase genes in the presence of high phosphate levels and constitutive mutations in Pho4p all map to a proline-rich region which interacts with Pho80p (Oshima, *et al.*, 1990; Jayaraman, *et al.*, 1994).

Studies in our lab and in the labs of others failed to uncover constitutive mutations in genes unlinked to the *MAL* loci (Needleman and Eaton, 1974; R. Needleman, 1970; Rodicio, 1986). Additionally, overexpression of *MAL63* using a high

copy vector (see Table 1), or by placing it under the control of the constitutive *ADH1* promoter or the inducible *GAL10* promoter did not result in constitutive expression of the *MAL* structural genes in the absence of maltose (Wojciechowicz, 1992). These results strongly suggest that a titratable Gal80p-like inhibitor is not a component of the *MAL* system, or if such a negative regulator is involved, it is an essential or highly abundant factor.

Residues 300-470 also are required for Mal63p induction. In-frame, internal 50-codon deletion mutations through this region resulted in nonfunctional LexA-Mal63p fusion proteins (Table 3). *mal63* nonsense mutations at codons 282, 307, 320, 333 and 456 are also noninducible (Chang *et al.*, 1989; Wojciechowicz, 1992; J. Kim, unpublished results). Although a stable protein product was demonstrated only for the 456 nonsense mutation, it is unlikely that all of the other noninducible mutant proteins are unstable (Wojciechowicz, 1992). Therefore, it appears that the C-terminal region of Mal63p provides a required function. The exact nature of this function remains undefined but some insight may be gained from recent studies in our laboratory. First, the requirement for this function is relieved in the *LexA-MAL63(1-283)* construction possibly as a result of the abundant expression of the LexA fusions from the *Saccharomyces* constitutive *ADH1* promoter. Additionally, constitutive mutations in *MAL64* were found to be nonsense mutations: *MAL64-C2* (nonsense

mutation at codon 307) and *MAL64-R10* (nonsense mutation at codon 282). It would appear that Mal64-C2p and Mal64-R10p are independent of the need for this C-terminal function. Overall Mal63p and Mal64p are 85% identical but, in residues 1-282 the differences are clustered to residues 51-91 (80% identical) and 236-260 (63% identical). The results reported here have defined the function of each of these regions. Analysis of the *trans*-activation ability of *MAL64/MAL63* chimeras is currently underway to define the functionally significant variant residues. Preliminary results suggest that the variations in the region identified as the activation domain in Mal63p are responsible for the functional difference between Mal64(1-283)p and Mal63(1-283)p. These studies should provide important clues to the function of the C-terminal required domain.

Deletion of residues 250-300 from LexA-Mal63p resulted in a constitutive activator of reporter gene expression but not of *MAL* gene expression. Examination of the sequence of this region reveals a proline-rich sequence, $A^{254}PPLPE^{259}$, which is similar to sequences identified in several proteins and believed to be involved in protein-protein interaction in cytoskeletal proteins (Donnelly *et al.*, 1993). Proline-rich regions have also been identified as required sequences in proteins that interact with the SH3 (Src-homology) domains of other proteins (Donnelly *et al.*, 1993). Deletion of this region in the yeast protein, Vrp1p, results in a temperature-

sensitive and pH-sensitive mutation, and cells carrying this allele have aberrant chitin and actin localization (Donnelly *et al.*, 1993). The importance of this region in the Mal63p is underscored by the existence of a noninducible *mal63* mutation, SW14, containing an alteration (Ala254 to Thr) in the invariant alanine residue found preceding the first proline in all of these proline-rich sequences examined. This region in Mal63p may represent a "hinge" region which links the regulatory region of the protein to the DNA-binding/activation region of the protein. Mutation of this region appears to uncover the activation function of the protein yet not allow it to function as a *MAL* gene activator. One interpretation of this result is that site-specific DNA-binding to the UAS_{MAL} is not occurring in these mutant proteins.

A model for maltose induction of Mal63p.

Based on the results presented here we would like to propose a model for the inducible maltose regulation of Mal63p. We suggest that, in the absence of maltose Mal63p is maintained in an inactive 3-dimensional conformation by the C-terminal negative regulatory domain (residues 300-470). In this conformational state (state I) either entry into the nucleus, DNA-binding and/or transcription activation does not occur. The addition of maltose brings about a structural change in the protein to conformational state II that now allows nuclear entry, DNA-binding and/or activation, all of

which functions lie in the N-terminal 283 residues. Residues in the C-terminal region from 250-470 are required in Mal63p to carry out this conformational change. On the other hand, residues 1-282 of Mal64p, while still sensitive to inhibition by the C-terminal regulatory domain in the absence of maltose are independent of the function provided by the C-terminal domain for conversion from structural state I to state II.

Residues 250-300 appear to play an important role in this conformational change, and the results with this deletion suggest that DNA-binding is being regulated by maltose. What is the function that the C-terminal domain provides? Perhaps some clues can be gleaned from the following. A search of the protein database revealed an interesting homology between Mal63p and the Chinese hampster topoisomerase II, an enzyme which undergoes extensive structural changes in the process of accomplishing its enzymatic function, the introduction of negative supercoils into DNA. Seven regions of homology to topoisomerase II were identified in Mal63p: residues 20-48 (31% identity, 41% similarity to topo II residues 1127-1155); residues 58-82 (43% identity, 56% similarity to topo II residues 111-133); residues 129-160 (21% identity, 48% similarity to topo II residues 365-397); residues 195-215 (35% identity, 55% similarity to topo II residues 503-522); residues 344-353 (50% identity, 90% similarity to topo II residues 547-556); residues 405-430 (29% identity, 59% similarity to topo II residues 1002-1025); and residues 440-

468 (39% identity, 46% similarity to topo II residues 1269-1296). Additionally, both topoisomerase II and Mal63p contain several potential casein kinase II phosphorylation sites, particularly in the C-terminal region. In the case of eukaryotic topoisomerase II, phosphorylation of these sites is required for enzymatic activation. The proposed model is that the C-terminal domain of topoisomerase II negatively regulates enzymatic activity and phosphorylation induces a conformational change needed to relieve the inactivation. Additionally, for enzyme activation to occur, a phosphorylation site is required at the C-terminal end of the protein since only those truncation mutations are active (Vassetzky *et al.*, 1994).

The homology to the MAL-activator is striking in one final respect. Several casein kinase II target sites can be found in Mal63p and Mal64p. Three of these conform very well to the ideal target sequence and are found at residues 267-273, 295-301 and 460-467 in Mal63p. Mal64p, which is inactive, is lacking the C-terminal CKII site. Both constitutive Mal64p mutations, *MAL64-C2* and *MAL64-R10*, are nonsense mutations which delete extensive sequences but which also position a CKII site at the C-terminus of the mutationally activated protein. *MAL64-C2* has a termination site at 307 just downstream of the 295-301 CKII site and *MAL64-R10* is truncated at codon 282 just downstream of the 267-273 CKII site (Wojciechowicz, 1992). While this evidence

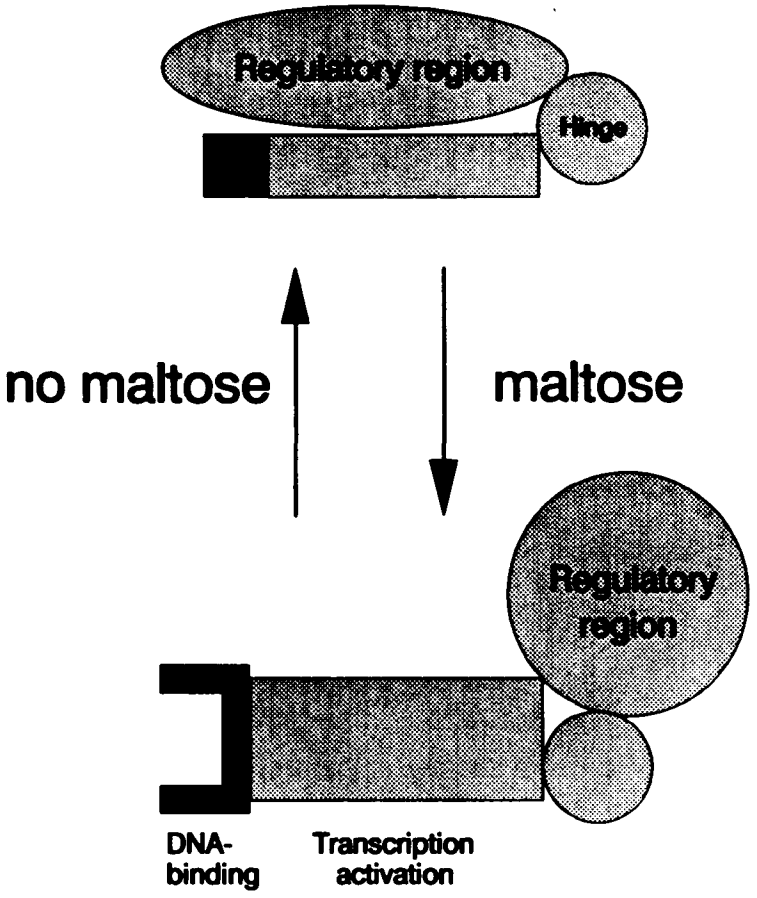
is very circumstantial, it might be suggesting that phosphorylation of the multiple CKII sites occurs during maltose-induction.

Phosphorylation has been shown to induce conformational changes in other proteins. Phosphorylation of a critical PK-A site in CREB induces a change in tertiary structure allowing this transcription activator to interact with the RNA-polymerase II complex (Gonzales *et al.*, 1991). The tumor suppressor p53 is also highly phosphorylated, undergoes conformational changes during activation and phosphorylation may be involved in the functional activation of this transcription factor (reviewed in Prives, 1994). Experiments are underway to test this model of MAL-activator induction.

Figure 5. Model of MAL-activator induction.

The DNA-binding domain, transcription activation region and maltose-responsive regulatory region are indicated. The regulatory region and DNA-binding domain are indicated as undergoing a conformational change from an inactive form to an active form in the absence or presence of maltose, respectively.

Model of MAL-activator Induction



Inactive form

The Regulatory region plays both a negative and a positive role in controlling MAL gene expression.
In the absence of maltose it inhibits the function of the MAL-activator.
In the presence of maltose it is required either for the process of induction or for the formation of the functional MAL-activator or both.

Active form

REFERENCES

Adams, B.G. 1973. Induction of galactokinase in *Saccharomyces cerevisiae*: kinetics of induction and glucose effects. J. Bacteriol. 111: 308-315.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl. 1989. Current protocols in molecular biology. John Wiley & Sons, NY.

Axelrod, J.D., J. Majors and M. Brandriss. 1991. Proline-independent binding of PUT3 transcriptional activator protein detected by footprinting in vivo. Mol. Cell. Biol. 11(1):564-567.

Bartel, P.L., C.T. Chien, R. Sternglanz, and S. Fields. 1993. Using the two-hybrid system to detect protein-protein interactions. In *Cellular Interactions in Development:A practical approach.*, D.A. Hartley, ed., Oxford University Press, Oxford;pp153-179.

Baichwal, V.R., A. Park and R. Tjian. 1992. The cell-type-specific activator region of c-jun juxtaposes constitutive and negatively regulated domains. Genes Dev. 6(8):1493-1502.

Beier, D.R. and E.T. Young. 1982. Characterization of a regulatory region upstream of *ADR2* locus of *S. cerevisiae*. Nature 300: 724-728.

Bemis, L.T. and C.L. Denis. 1988. Identification of functional regions in the yeast transcriptional activator Adr1. Mol. Cell. Biol. 8: 2125-2131.

Berger, S.L., B. Piña, N. Silverman, G.A. Marcus, J. Agapite, J.L. Regier, S.J. Triezenberg and L. Guarente. 1992. Genetic isolation of ADA2: A potential transcriptional adaptor required for function of certain acidic activation domains. Cell 70:251-265.

Blumberg, H. T.A. Hartshorne, and E.T. Young. 1988. Regulation of expression and activity of the yeast transcriptional factor Adr1. Mol. Cell. Biol. 8: 1868-1876.

Blumberg, H., A. Eisen, A. Sledziewski, D. Bader, and E.T. Young. 1987. Nuclear Localization of Adr1p. Nature 328:443-445.

Bohen, S.P., and Yamomoto, K.R. 1993. Isolation of Hsp90 mutants by screening for decreased steroid receptor function. Proc. Natl. Acad. Sci. USA 90: 11424-11428.

Branden, C. and J. Tooze. 1991. Introduction to protein structure. Garland Publishing, Inc. NY.

Brisco, P.R.G., T.S. Cunningham and G. B. Kohlhaw. 1987. Cloning, disruption and chromosomal mapping of yeast *LEU3*, a putative regulatory gene. Genetics 115: 91-99.

Chang, Y.S., R.A. Dubin, E. Perkins, D. Forrest, C.A. Michels, and R.B. Needleman. 1988. *MAL63* codes for a positive regulator of maltose fermentation in *Saccharomyces cerevisiae*. Curr. Genet. 14: 201-209.

Charron, M.J., and C.A. Michels. 1987. The constitutive glucose-repression-insensitive mutation of the yeast *MAL4* locus is an alteration of the *MAL43* gene. Genetics 116: 23-31.

Charron, M.J., E. Read, S.R. Haut and C.A. Michels. 1989. Molecular evolution of the telomere-associated *MAL* loci of *Saccharomyces*. Genetics 134: 261-272.

Cheng,Q. and C.A. Michels. 1989. The maltose permease encoded by the *MAL61* gene of *Saccharomyces cerevisiae* exhibits both sequence and structural homology to other sugar transporters. Genetics 123: 477-484.

Cherry, J.R., T.R. Johnson, C. Dollard, J.R. Shuster, and C. L. Denis. 1989. Cyclic AMP-dependent protein kinase phosphorylates and inactivates the yeast transcriptional activator Adr1. Cell 56: 409-419.

Ciriacy, M. 1979. Isolation and characterization of further cis- and trans-acting regulatory elements involved in the synthesis of glucose repressible alcohol dehydrogenase (*ADH11*) in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 176: 427-431.

Cook, W.J., S.P. Mosley, D. Audino, D.L. Mullaney, A. Rovelli, G. Stewart, and C.L. Denis. 1994. Mutations in the zinc-finger region of the regulatory protein ADR1 affect both DNA-binding and transcriptional activation. J. Biol. Chem. 269(12): 9374-9379

Cook, W.J., D. Chase, D.C. Audino, and C.L. Denis. 1994. Dissection of the ADR1 protein reveals multiple, functionally redundant activation domains interspersed with inhibitory regions:evidence for a repressor binding to the ADR1^{c} region. Mol. Cell. Biol. 14(1):629-640.

Corton, J. C., and S.A. Johnston. 1989. Altering DNA-binding specificity of GAL4 requires sequences adjacent to the zinc finger. Nature 340:724-727.

Defranoux, N., M. Gaisne, and J. Verdiere. 1994. Functional analysis of the zinc cluster domain of the CYP1(HAP1) complex regulator in heme-sufficient and heme-deficient yeast cells. Mol. Gen. Genet. 242: 699-707

Denis, C.L., S.C. Fontaine, D. Chase, B.E. Kemp and L.T. Bemis. 1992. *ADR1*^{c} mutations enhance the ability of ADR1 to activate transcription by a mechanism that is independent of effects on cyclic AMP-dependent protein kinase phosphorylation of Ser-230. Mol. Cell. Biol. 12(4):1507-1514.

Denis, C.L., M. Ciriacy, and E.T. Young. 1981. A positive regulatory gene is required for accumulation of the functional mRNA for the glucose-repressible alcohol dehydrogenase from *Saccharomyces cerevisiae*. J. Mol. Biol. 148: 355-368.

Denis, C.L. 1984. Identification of new genes involved in the regulation of yeast alcohol dehydrogenase 11. Genetics 108: 833-844.

Denis, C.L. 1987. The effects of *ADR1* and *CCR1* gene dosages on the regulation of the glucose repressible alcohol dehydrogenase from *Saccharomyces cerevisiae*. Mol. Gen. Genet. 208: 101-106.

Devereux, J., P. Haeberle and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids Res. 12:387-395.

Donnelly, S.F.H., M.J. Pockington, D. Pallotta and E. Orr. 1993. A proline-rich protein, verprolin, involved in cytoskeletal organization and cellular growth in the yeast *Saccharomyces cerevisiae*. Molec. Microbiol. 10: 585-596.

Douglas,H.C., and D.C. Hawthorne. 1966. Regulation of genes controlling synthesis of the galactose pathway enzymes in yeast. Genetics 54: 911-916.

Dubin, R.A., R. B. Needleman, D. Gossett and C.A. Michels. 1985. Identification of the structural gene encoding maltase within the *MAL6* locus of *Saccharomyces cerevisiae*. J. Bacteriol. 164:605-610.

Dubin,R.A., M.J. Charron, S.R. Haut, R.B. Needleman and C.A, Michels. 1988. Constitutive expression of the maltose fermentative enzymes in *Saccharomyces carlsbergensis* is dependent upon the mutational activation of a non-essential homolog of *MAL63*. Mol. Cell. Biol. 8: 1027-1035.

Evans, R.M. 1988. The steroid and thyroid hormone receptor

superfamily. Science 240: 889-985.

Fields, S., and R. Sternglanz. 1994. The two-hybrid system: an assay for protein-protein interactions. TIGS 10(8):286-292.

Fytlovich, S., M. Gervais, C. Agrimonti and B. Guiard. 1993. Evidence for an interaction between the CYP1(HAP1) activator and a cellular factor during heme-dependent transcriptional regulation in the yeast *Saccharomyces cerevisiae*. EMBO J. 12:1209-1218.

Ge, H. and R. G. Roeder. 1994. Purification, cloning and characterization of a human coactivator, PC4, that mediates transcriptional activation of class II genes. Cell 78:513-523.

Giniger, E., S. Varnum, and M. Ptashne. 1985. Specific DNA binding of Gal4, a positive regulatory protein of yeast. Cell 40: 767-774.

Godowski, P.J., S. Rusconi, R. Miesfeld, and K.R. Yamamoto. 1987. Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. Nature 325: 365-368.

Gonzalez, G.A., P. Menzel, J. Leonard, W.H. Fischer, and M.R. Montminy. 1991. Characterization of motifs which are critical for activity of the cyclic AMP-responsive transcription factor CREB. Mol. Cell. Biol. 11(3): 1306-1312.

Guarente, L., R.R. Yocum, and P. Gifford. 1982. A Gal10-Cyc1 hybrid yeast promoter identifies the *GAL4* regulatory region as an upstream site. Proc. Natl. Acad. Sci. USA 79: 7410-7414.

Guarente, L. 1984. Regulatory proteins in yeast. Ann. Rev. Genet. 21: 425-452.

Hill, J., K.A. Donald, D.E. Griffiths and G. Donald. 1991. DMSO-enhanced yeast transformation. Nucleic Acids Res. 19(20):5791-5792.

Hill, J.E., A.M. Meyers, T.J. Koerner and A. Tzagaloff. 1986. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast 2:163-167.

Hollenberg, S.M., and R.M. Evans. 1988. Multiple and cooperative *trans*-activation domains of the human glucocorticoid receptor. Cell 55:899-906.

Hong, S.H., and J. Marmur. 1986. The primary structure of the maltase gene of the *MAL6* locus of *S. Carlsbergensis*.

Gene 41: 75-84.

Hopper, J.E., J.R. Broach, and L.B. Rowe. 1978. Regulation of the galactose pathway in Saccharomyces cerevisiae: induction of uridyl transferase mRNA and dependency on *GAL4* gene function. Proc. Natl. Acad. Sci. USA. 75: 2878-2882.

Howard, K.J., and C.W. Distelhorst. 1988. Evidence for intracellular association of the glucocorticoid receptor with the 90-kDa heat shock protein, J. Biol. Chem. 263:3474-3481.

Ito, H., Y. Fukuda, K. Murata and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153:163-168.

Jayaraman, P-S., K. Hirst, and C.R. Gooding. 1994. The activation domain of a basic helix-loop-helix protein is masked by repressor interaction with domains distinct from that required for the transcription regulation. EMBO J. 13(9):2192-2199.

Johnston, M. 1987. A model fungal gene regulatory circuit: The *GAL* genes of *Saccharomyces cerevisiae*. Microbiol. Rev. 51: 458-476.

Johnson, S.A., and J.E. Hopper. 1982. Isolation of the yeast regulatory gene *GAL4* and analysis of its dosage effects on the galactose/melibiose regulon. Proc. Natl. Acad. Sci. USA 79: 6971-6975.

Johnston, S.A., J.M. Salmeron Jr., and S.S. Dincher. 1987. Interaction of positive and negative mutations that inactivate a yeast transcriptional regulatory protein in the galactose regulon of yeast. Cell 50: 143-146.

Karin, M. 1990. Too many transcription factors: Positive and negative interactions. The New Biologist. 2: 126-131.

Keegan, L., G. Gill, and M. Ptashne. 1986. Separation of DNA binding from activating function of a eukaryotic regulatory protein. Science 231: 699-704.

Keleher, C.A., M.J. Redd, J. Schultz, M. Carlson and A.D. Johnson. 1992. Ssn6-Tup1 is a general repressor of transcription in yeast. Cell 68:709-719.

Kelly, R. and K.J. Kwon-Chung. 1992. A zinc finger protein from *Candida albicans* is involved in sucrose utilization. J. Bacteriol. 174(1):222-232.

Kim, B., and J. W. Little. 1992. Dimerization of a specific DNA binding protein on the DNA. Science 255: 203-206.

Kim, J., and C.A. Michels. 1988. The *MAL63* gene of *Saccharomyces* encodes a cysteine-zinc finger protein. Curr. Genet. 14: 319-323.

Kim, K.S., and L. Guarente. 1989. Mutations that alter transcriptional activation but not DNA binding in the zinc finger of yeast activator Hap1. Nature 342: 200-203.

Kim, K.S., K. Pfeifer, L. Powell and L. Guarente. 1990. Internal deletions in the yeast transcriptional activator Hap1 have opposite effects at two sequence elements. Proc. Natl. Acad. Sci. USA 87: 4524-4528.

Kim, J.H. 1992. Ph.D. Thesis. City University of New York.

Khan, N.A., and N.R. Eaton. 1971. Genetic control of maltase formation in yeast. 1. Strains producing high and low basal levels of enzyme. Mol. Gen. Genet. 112: 317-322.

Kirkman-Correia, C., I.L. Stroke and S. Fields. 1993. Functional domains of the yeast STE12 protein, a pheromone-responsive transcriptional activator. Mol. Cell. Biol. 13(6):3765-3772.

Kretzschmar, M., K. Kaiser, F. Lottspeich and M. Meisterernst. 1994. A novel mediator of class II gene transcription with homology to viral immediate-early transcriptional regulators. Cell 78:525-534.

Lemire, J.M., T. Willcock, H.O. Halvorson, and K.A. Bostian. 1985. Regulation of repressible acid phosphatase gene transcrription in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 5: 2131-2141.

Leuther, K.K., and S.A. Johnston. 1992. Nondissociation of GAL4 and GAL80 *in vivo* after galactose induction. Science 256:1333-1335.

Leuther, K.K., J. M. Salmeron and S.A. Johnston. 1993. Genetic evidence that an activation domain of GAL4 does not require acidity and may form a β sheet. Cell 72:575-585.

Levine, J., L. Tanouye, and C. Michels. 1992. The UAS_{MAL} is a bidirectional promoter element required for the expression of both the *MAL61* and *MAL62* genes of the *Saccharomyces* MAL6 locus. Curr. Genet. 22:181-189.

Lewin, B. 1990. Commitment and activation at Pol 11 promoters: a tail of protein-protein interactions. Cell 61:1161-1164.

Li, F-Q., H. Ueda, and S. Hirose. 1994. Mediators of

activation of fushi tarazu gene transcription by BmFTZ-F1. Mol. Cell. Biol. 14:3013-3021.

Ma, J., and M. Ptashne. 1987b. The carboxyl-terminal 30 amino acids of Gal4 are recognized by Gal80. Cell 50: 137-142.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Marczak-DaCosta, Judith, E. 1990. The Yeast Put3 transcriptional activator: Molecular, Mutational and sequence analysis. Ph.D. Thesis. University of Medicine and Dentistry of New Jersey.

Marczak, J.E. and M. Brandriss. 1991. Analysis of constitutive and noninducible mutations of the PUT3 transcriptional activator. Mol. Cell. Biol. 11(5):2609-2619.

Mitchell, P.J., and R. Tjian. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245:371-378.

Mylin, L.M., J.P. Bhat, and J.E. Hopper. 1989. Regulated phosphorylation and dephosphorylation of Gal4, a transcriptional activator. Genes and Dev. 3: 1157-1165.

Mylin, L.M., M. Johnston, and J.E. Hopper. 1990. Phosphorylated forms of Gal4 are correlated with ability to activate transcription. Mol. Cell. Biol. 10:

Needleman, R.B. 1970. Ph.D. thesis. The City University of New York.

Needleman, R.B. and C. Michels. 1983. Repeated family of genes controlling maltose fermentation. Mol. Cell. Biol. 3:796-802.

Needleman, R.B., D.B. Kaback, R.A. Dubin, E.L. Perkins, N.G. Rosenberg, K.A. Sutherland, D.B. Forrest, and C.A. Michels. 1984. *MAL6* of *Saccharomyces*: A complex genetic locus containing three genes required for maltose fermentation. Proc. Natl. Acad. Sci. USA. 81: 2811-2815.

Ni,B., and R.B. Needleman, 1990. Identification of the upstream activating sequence of *MAL* and binding sites for the *MAL63* activator of *Saccharomyces cerevisiae*. Mol. Cell. Biol. 10: 3997-3800.

Ogawa, N., and Y. Oshima. 1990. Functional domains of a positive regulatory protein, Pho4, for transcriptional

control of the phosphatase regulon in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 10: 2224-2236.

Okada, H. and A. Toh-e. 1992. A novel mutation occurring in the *PHO80* gene suppresses the *PHO4*^{c} mutations of Saccharomyces cerevisiae. Curr. Genet 21:95-99.

Oshima, Y. 1982. Regulatory circuits for gene expression: the metabolism of galactose and phosphate, p159-180. in J.N. Strathern, E.W. Jones and J.R. Broach (eds.) *The Molecular Biology of the Yeast Saccharomyces: metabolism and gene expression*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Parthum, M. R. and Jaehning, J.A. 1992. A transcriptionally active form of GAL4 is phosphorylated and associated with GAL80. Mol. Cell. Biol. 12:4981-4987

Pfeifer, K., T. Prezant, and L. Guarente. 1987. Yeast Hap1 activator binds to two upstream activation sites of different sequences. Cell 49: 19-27.

Pfeifer, K., K.S. Kim, S. Kogan, and L. Guarente. 1989. Functional dissection and sequence of yeast Hap1 activator. Cell 56: 291-301.

Picard, D., S.J. Salser, and K.R. Yamamoto. 1988. A movable and regulable inactivation function within the steroid binding domain of the glucocorticoid receptor. Cell 54: 1073-1080.

Piña, B., S. Berger, G.A. Marcus, N. Silverman, J. Agapite and L. Guarente. 1993. ADA3: a gene, identified by resistance to GAL4-VP16, with properties similar to and different from those of ADA2. Mol. Cell. Biol. 13(10):5981-5989.

Prives, C. 1994. How loops, β-sheets, and α-helices help us to understand p53. Cell 78:543-546.

Ptashne, M. 1978. *The Operon*, p.325-344. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Reece, R.J. and M. Ptashne. 1993. Determinants of binding-site specificity among yeast C_6 zinc cluster proteins. Science 261:909-911.

Rihs, H.P., D.A. Jans, H. Fan, and R. Peters. 1991. The rate of nuclear cytoplasmic protein transport is determined by the casein kinase II site flanking the nuclear localization sequence of SV40 T-Antigen. EMBO 10:633-639.

Ruden, D.M., J. Ma, Y. Li, K. Woos ans M. Ptashne. 1991.

Generating yeast transcriptional activators containing no yeast protein sequences. Nature 350:250-252.

Salmeron, J.M., K.K. Leuther and S.A. Johnston. 1990. *GAL4* mutations that separate the transcriptional activation mutations and *GAL80* interactive functions of the Gal4 protein. Genetics 125: 21-27.

Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

Schneider, K. R., R.L. Smith and E.K. O'Shea. 1994. Phosphate- regulated inactivation of the kinase PHO80-PHO85 by the CDK inhibitor PHO81. Science 266:122-126.

Sherman, F., G.R. Fink, and J.B. Hicks. 1986. Laboratory course manual for Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sikorski, R.S. and P. Heiter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122:19-27.

Song, O-K., J.W. Dolan, Y.O. Yuan, and Stanley Fields. 1991. Pheromone-dependent phosphorylation of the yeast STE12 protein correlates with transcriptional activation. Genes & Dev. 5:741-750.

Stanway, C.A. 1993. Simplicity amidst complexity in transcriptional iniation. Bioessays 15(8): 559-560.

Sze, J-Y., M. Wootner, J.A. Jaehning, and G.B. Kohlhaw. 1992. *In vitro* transcriptional activation by a metabolic intermediate: Activation by Leu3 depends on α-isoprorypmalate. Science 258:1143-1145.

Sze, J-Y., E. Remboutsika and G.B, Kohlhaw. 1993. Transcriptional regulator Leu3 of Saccharomyces cerevisiae: Separation of activator and repressor functions. Mol. Cell. Biol. 13(9):5702-5709.

Taylor, W. E., and E. T. Young. 1990. cAMP-dependent phosphorylation and inactivation of yeast transcription factor Adr1 does not affect DNA binding. Proc. Natl. Acad. Sci. USA 87: 4098-4102.

ten Berge, A.M.A., G. Zoutewelle, K. W. van de Poll, H.P.J. Bloemers. 1973. Regulation of maltose fermentation in *Saccharomyces carlsbergensis* 11. Properties of a constitutive *MAL6* mutant. Molec. gen. Genet. 125: 139-146.

ten Berge, A.M.A., G. Zoutewelle and K.W. Van de Poll. 1973. Regulation of maltose fermentation in *Saccharomyces carlsbergensis*. I. The function of the gene MAL_6, as recognized by mal_6-mutants. Mol. Gen. Genet. 123:233-246

Thill, G.P., R.A. Kramer, K.J. Turner, and K. A. Bostian. 1983. Comparative analysis of the 5'-end regions of two repressible acid phosphatase genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 3: 570-579.

Tjian, R. and Maniatis, T. 1994. Transcriptional activation: A complex puzzle with few easy pieces. Cell 77: 5-8.

Van Hoy, M., K.K. Leuther, T. Kodadek, and S.A. Johnston. 1993. The acidic activation domains of the GCN4 and GAL4 proteins are not α-helical but form β-sheets. Cell 72:587-594.

Vassetzky, Y.S., Q. Dang, P. Benedetti and S.M. Gasser. 1994. Topoisomerase II forms multimers in vitro: Effects of metals β-glycerophosphate, and phosphorylation of its C-terminal domain. Mol. Cell. Biol. 14(10):6962-6974.

Walker, S., R. Greaves and P. O'Hare. 1993. Transcriptional activation by the acidic domain of Vmw65 require the integrity of the domain and involves additional determinants distinct from those necessary for TFIIB binding. Mol. Cell. Biol. 13(9):5233-5244.

Wojciechowicz, L.A. 1993. Ph.D. Thesis. The City University of New York.

Yoshida, K., Z. Kuromitsu, N. Ogawa, and Y. Oshima. 1989. Function of *PHO* regulatory genes for repressible acid phosphatase synthesis in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 217: 40-46.

Zhang, L., O. Bermingham-McDonogh, B. Turcotte, and L. Guarente. 1993. Antibody-promoted dimerization bypasses the regulation of DNA binding by the heme domain of the yeast transcriptional activator HAP1. Proc. Natl. Acad. Sci. USA 90:2851-2855.

Zhang, L. and L. Guarente. 1994. HAP1 is nuclear but is bound to a cellular factor in the absence of heme. Jour. Biol. Chem. 269(20): 14643-14647.

Zhou, K., Y. Bai, and G.B. Kohlhaw. 1990. Yeast regulatory protein Leu3: a structure-function analysis. Nuc. Acids Res. 18(2): 291-298.

Zhou, K. and G.B. Kohlhaw. 1990. Transcriptional activator

LEU3 of yeast. J. Biol. Chem. 265(29): 17409-17412.

Zimmerman, F.K., and N.R. Eaton. 1974. Genetics of induction and catabolite repression of maltase synthesis in *Saccharomyces cerevisiae*. Molec. Gen. Genet. 134: 261-272.